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A FACTOR IN OLD HEPATITIS SERUM CAPABLE OF AGGLUTINATING CHICKEN RED CELLS*

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With the technical assistance of MISS MARIE A. FOARD

Received for publication February 7, 1949

During the course of a study of the agglutination of red cells previously treated with Newcastle Virus (1), it was discovered that certain hepatitis sera in a very high dilution were capable of agglutinating normal washed red blood cells from certain chickens. Since these sera were often acute phase sera† the question arose whether the agglutinating factor might be the virus of infectious hepatitis. Although our results indicate that the agglutinating factor is not the virus, the nature of the problem makes it impossible to state this with certainty. It was further demonstrated that the reaction of agglutination or pattern formation could be produced with red cells from only certain chickens, and that the degree of reaction could be partially influenced by the method of washing the glassware. Fresh hepatitis serum did not agglutinate these red cells. Cells exposed to this factor did not become resistant to several of the viruses capable of agglutinating chicken red cells.

METHOD

Tests for agglutination were performed by making serial twofold dilutions of the serum in a one per cent phosphate buffered saline of .14 molarity (pH 7.2). Dilutions were made in Wassermann tubes and the end point judged by the pattern which formed at the bottom of the tube as the red cells settled in 45–60 minutes at room temperature (20–25°C.). Several tests run simultaneously in the icebox, incubator, and at room temperature showed no difference. Routinely, all glassware was first cleaned mechanically by rubbing with a test tube brush

* Supported in part by a grant in aid from the Nat. Inst. of Health of the U.S.P.H.S. and conducted in part under contract with the Office of Naval Research, U. S. Navy.

† Sera taken during the acute phase of the hepatitis.

without soap, then terminally cleaned by immersion in dichromate cleaning solution for 24 hours. It was finally rinsed five times in tap water and twice in distilled water. Chicken red cells were obtained by washing citrated blood with one per cent phosphate buffered saline (pH 7.2) two times. A final concentration of about one per cent of packed cells was used. No red cell preparations older than a week were used. The old sera studied here were serial bleedings obtained by Dr. George S. Mirick from cases of acute hepatitis in Guam and at Rockefeller Institute, New York (1944–1945). They had been stored at about 4°C. since then. Only clear serum was tested. Cultures for aerobic and anaerobic organisms and fungi were done on 29 negative agglutinators, five of which were positive, and on 8 positive agglutinators, 4 of which were positive.

The first serum found capable of agglutinating normal chicken red blood cells was highly specific in that only about one half of the preparations of red cells obtained from different chickens were capable of any agglutination by this serum. Red cells obtained from the positive chickens were repeatedly and uniformly agglutinated by that particular serum in dilutions of 1/5000 or more. It was subsequently found that when a chicken furnished red cells capable of agglutination by one positive serum, its red cells would usually be agglutinated by any other positive serum (Table I). That this specificity of cells and serum had however nothing to do with blood types in man is likely because different sera from the same individual patient failed to agglutinate the same red cells. This specificity is reminiscent of that described for vaccinia and ectromelia hemagglutination (2), and in the light of the known individual differences in chicken blood (3) is not surprising.

Following this preliminary characterization of the reaction, the study was enlarged and Dr. Mirick undertook to check the ability of a number of other hepatitis sera to agglutinate these red cells. He obtained positive results with sera which in our hands had been negative with the same red cell-serum combination. It was then discovered that the type of cleaning and washing to which the glassware had been subjected was the cause of the difference in results and that a serum which was negative in tubes previously washed with cleaning solution was positive in tubes washed in surgical green soap before

rinsing. That the effect of the soap was a potentiating one and very persistent may be seen by the large number of times that it was necessary to rinse the tubes to get rid of this effect.

Not only was the type of washing capable of making certain negative sera positive but sera which were capable in a dilution of 1/800 of

TABLE I

Agglutination of Washed Chicken Red Cells by Old Sera in Dilution of 1/100 or More

CELLS FROM CHICKEN #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
63		0		0	0										
53		0			0										
134		0													
168						0									
190	0					0									
11		0			0										
26															
76		0		0	0										
5		0		0	0										
195		0													
165		0													
105	+		+			+									
174	+					+									
138	0	+													
125		+													
122	+	+													
119		0	+												
112	+	+													
92		+			0	+	0	0	0	0	0	0	+	0	0
31				+											
26		+		+											
8		+		+	0	0	0	0	0	0	0	0	0	0	0
3		+		+	0	0									
64	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

agglutinating red cells were made more powerful by titration in soap-washed tubes (Table III).

Attempts were, therefore, made to run many of the tests in both soap-washed and acid washed tubes. This established the potentiating effect of the previous treatment with soap, but the difficulty of exactly reproducing the amount of soap left behind in each tube led us to add graded amounts of soap to the different tubes. In this way it was

found that a 1/80,000 dilution of a concentrated suspension of green surgical soap potentiated the ability of certain sera to agglutinate chicken red cells (Table IV). Higher concentrations either agglutinated all cells or hemolyzed them.

TABLE II

Effect of Type of Washing and Number of Rinsings on the Red Cell Agglutination Produced by Serum Negative in Acid Cleaned Tubes

	NUMBER OF RINSINGS		RESULTS									
			Dilution of serum									
	Tap water	Distilled water	1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400	1/12800	1/25600	1/51200
Soap cleaned	2	0	0	+++	+++	+++	+++	+++	++	+	0	0
	5	2	+++	+++	+++	+++	+++	++	0	0	0	0
	10	10	0	0	0	0	0	0	0	0	0	0
Acid cleaned	2	0	0	0	0	0	0	0	0	0	0	0
	5	2	0	0	0	0	0	0	0	0	0	0
	10	10	0	0	0	0	0	0	0	0	0	0

TABLE III

Potentiating Effect of Soap Washed Tubes on Agglutination by a Serum Positive in Acid Cleaned Tubes

	NUMBER OF RINSINGS		DILUTION OF SERUM									
	Tap water	Distilled water	1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400	1/12800	1/25600	1/51200
Soap cleaned	2	0	+++	+++	+++	+++	+++	+++	+++	+++	++	++
	5	2	+++	+++	+++	+++	+++	+++	+++	++	++	0
	10	10	+++	+++	+++	+++	+++	+++	0	++	0	0
Acid cleaned	2	0	+++	+++	+++	+++	++	+	0	0	0	0
	5	2	+++	+++	+++	+++	++	++	0	0	0	0
	10	10	+++	+++	+++	+++	++	+	0	0	0	0

It is interesting to note in this table that the presence of soap did not make cells (# 5) which were not ordinarily agglutinated by positive sera, capable of agglutination. This would indicate a degree of specificity of effect. It was, however, true that larger concentrations of soap were capable in themselves of agglutinating normal chicken

red cells, and because of this and the difficulty of controlling the reaction, we continued our studies using only tubes cleaned in acid and routinely washed three times in tap water and twice in distilled water. All results reported subsequently in this paper are then from such tubes.

In a limited number of experiments we attempted to partially characterize the factor in the serum which is capable of agglutinating the chicken red cells. For this only strongly positive sera were used.

TABLE IV

Effect of Minute Amounts of Soap Added to the Serum Before the Addition of Red Cells

RED CELLS FROM CHICKEN #	AMOUNT OF SOAP FINAL CONC.	SERUM DILUTION									
		1/50	1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400	1/12800	1/25600
92	1/80,000	0	+	++	++	++	++	0	0	0	0
	1/160,000	0	+	+	++	++	0	0	0	0	0
	None	0	0	0	0	0	0	0	0	0	0
8	1/80,000	+	++	+++	+++	+++	+++	++	+	0	0
	1/160,000	0	0	++	+++	+++	+	0	0	0	0
	None	0	+	+	++	+	+	++	0	0	0
5	1/80,000	0	0	0	0	0	0	0	0	0	0
	1/160,000	0	0	0	0	0	0	0	0	0	0
	None	0	0	0	0	0	0	0	0	0	0

Size of particle or molecules responsible for agglutination.—Assuming a density greater than water, larger molecules should be sedimented in a centrifugal field. If the agglutination here studied was caused by a virus of the usual density it might be expected that a force of approximately 50–90,000 g. for one hour in a physiological salt solution would produce a change in the distribution of particles of a size of 50 mμ or larger.

In two tests run in a Sorval angle centrifuge at about 13,000 r.p.m. (17,000 g.) and one test in the air-driven ultracentrifuge at the Rockefeller Institute for Medical Research at Princeton, N. J. at 24,000 r.p.m. (50–90,000 g.) there was no evidence of sedimentation. This latter centrifuge has been repeatedly used for the sedimentation of equine encephalomyelitis which is about 50 mμ in size (4, 5).

METHOD

Serum was diluted 1/10 in normal saline and the centrifugation carried out in a pre-cooled head (4°C.). Two separate sera were tested, one with a titer of 1:3200, the other 1:200. After centrifugation for one hour the top one cc. was taken carefully off, then a sample from the middle, and finally the bottom fluid was mixed with the film which had collected at the bottom of the tube. In no case was the titer of the top sample after centrifugation below that of the original fluid, and the titer of the pellet resuspended in the fluid at the bottom at no time indicated any increase of red cell agglutinating activity.

These results indicate that the factor or particle causing the agglutination is less than 50 mμ but in the absence of data on the density of this component no further study was practicable in this laboratory.

On the other hand, in three separate experiments, dialysis through a viscose collodion sac of two positive sera failed to demonstrate the passage of any of the red cell agglutinating factor through the pores of the collodion sac. Again care was taken to use sera with a high titer (1/600–1/3200) and this was diluted 1/10 in saline before dialysis. This might indicate that its "molecular weight" is above 6,000, since trypsin inhibitor, which has a molecular weight of about 6,000, will diffuse through such membranes (6).

Crude fractionation of the serum by dilution in distilled water showed that the agglutinating factor could be precipitated and recovered on resuspension in saline, but that the greater fraction was left behind in the supernatant (Table V). Precipitation of serum with both 50 per cent and 75 per cent ammonium sulphate brought about a concentration of the factor in the precipitate in both instances.

Relation to Hepatitis.—The great need for a practical test for the presence of the virus of infectious hepatitis in serum led us to inquire carefully into the possibility that the reaction here studied was caused by that virus:

1. Table VI shows that positive sera were found throughout the course of infectious hepatitis and that high titers were obtained after the acute disease was over. Positive reactions were more frequent early in the course of the illness. The positive results obtained in late convalescence would not in themselves rule out a viral etiology of the phenomenon (7).

2. A comparison of the "neutralizing ability" of acute serum with serum taken one to two months later in 6 cases of infectious hepatitis has failed to show any change in the "neutralization" of 10 hemagglutinating doses of the agent. This would tend to exclude the virus

TABLE V

Precipitation of Agglutinating Factor From Sera on Dilution in Distilled Water (Red Cell Tests done in Saline)

DIL. IN WATER		1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10240	1/20480	1/40960
1/2	Precip.	+++	+++	+++	+++	+++	+++	+	+	0	0		
	Supernat.	h	+++	+++	+++	+++	++	++	++	++	0		
1/5	Precip.	h	+++	+++	+++	+++	+++	++	+	0	0		
	Supernat.	h	h	+++	+++	+++	+++	+++	++	+	+		
1/10	Precip.	+++	+++	+++	+++	+++	0	0	0	0	0	0	0
	Supernat.	h	h	+++	+++	+++	+++	+++	++	0	0	0	0
1/10	Precip.		+++	+++	+++	+++	+	0	0	0	0	0	0
	Supernat.		h	h	+++	+++	+++	+++	++	+	0	0	0
1/20	Precip.	+++	+++	+++	+++	+	0	0	0	0	0	0	0
	Supernat.			h	+++	+++	+++	+++		0	0	0	0

Note: Dilution of Precipitate above is on the basis of original volume of serum although the small volume of precipitate probably was less than 1/50 of the original volume.

TABLE VI

Relation of Day of Disease to Subsequent Hemagglutinization

DAY OF ILLNESS	# TESTED	# POSITIVE	%
0–15	19	8	42.1
16–30	27	2	7.4
30–45	16	2	12.5
36+	16	2	12.5

from consideration but again in the absence of basic knowledge concerning humoral antibodies in this disease, we cannot be sure (7).

If the hemagglutination were due to the virus of infectious hepatitis, one might expect that fresh sera from cases of infectious hepatitis would be equally capable of agglutinating red cells. The work on the

masking of the hemagglutinating capacity of the pneumonia virus of mice (8) is a clear example, however, of how a negative material can after certain treatment become positive, and thus we cannot exclude the possibility that this hemagglutination is of viral etiology even though none of 22 fresh sera from 11 individuals with infectious hepatitis agglutinated suitable chicken red cells.

Satisfactory controls on sera which have been stored for periods of time at ordinary but slightly variable refrigeration are difficult to obtain. Of thirty sera collected in 1942–1945 from 10 cases of primary atypical pneumonia at the Rockefeller Institute one agglutinated chicken cells in a dilution of 1/200. On the other hand, of 20 sera

TABLE VII

Tests for Agglutination in Other Sera

KIND OF CASE	# PATIENTS	# SERA	# POSITIVE
Infectious hepatitis, fresh sera	11	22	0
Infectious hepatitis, old sera, Rockefeller Inst.	10	20	0
Infectious hepatitis, old sera, Johns Hopkins Hospital	3	9	2
Non-infectious jaundice, old sera, Johns Hopkins Hospital	9	16	1
Primary atypical pneumonia, old sera, Rockefeller Inst.	10	30	1

from 10 cases of infectious hepatitis, obtained at the same place and time, none agglutinated chicken red cells.[1]

Of sixteen sera, about a year old, from nine cases of non-infectious jaundice at the Johns Hopkins Hospital, only one was positive, this a grossly contaminated serum which would have ordinarily been excluded. Of nine one-year old sera from 3 cases of infectious hepatitis, 2 agglutinated chicken red cells in a dilution of 1/80 and 1/160. This data is summarized in Table VII, and unfortunately does not give an unequivocal answer to the question of virus hemagglutination.

DISCUSSION

One obvious possibility which has not been dealt with in the text of this paper is that contamination of the sera with bacteria or fungi was

[1] Courtesy Dr. F. L. Horsfall.

the cause of the agglutination. This remains a likely explanation but should not be used as the final answer. Only clear sera were used. Of 8 sera which subsequently were shown to contain agglutinins and which were cultured before any work was done, 4 or 50 per cent yielded bacterial growth on sub-culture into Brewer's thioglycollate media and/or on a blood plate. Of 29 sera subsequently shown to be negative for agglutinins, 5 yielded bacteria. In another case an unidentified fungus was found. This latter, in one test, produced red cell agglutinins when grown in normal serum. However, it is well-known that a number of bacteria will agglutinate different red cells and, as indicated above, a number of sera which had a high content of agglutinins were negative on culture. It is true that in these latter sera the bacteria might have died out and the agglutinins have persisted.

Despite this it was felt that the high titer of the substance in the sera, the limitation of the reaction to red cells derived from only certain chickens, and the effect of the type of cleaning of glassware on the reaction justify the report.

SUMMARY

A factor capable of agglutinating chicken red blood cells was found in old hepatitis sera. This factor, which would agglutinate only cells from certain chickens, was present in high concentrations, particularly during the first fifteen days of illness. During this time, 8 of 19 (42 per cent) sera were positive in titers of 1/600 to 1/15,000. Thereafter a total of 59 sera yielded six that were positive (10 per cent) with a titer of 1/60 to 1/1260. The pattern agglutination of these chicken red cells was potentiated by traces of soap such as may be left behind after inadequate rinsing but the reaction was regularly obtained in tubes cleaned in chromic acid cleaning solution which was the standard used throughout. The factor was not directly related to the original bilirubin level of the plasma nor to the occasional bacterial or fungous contaminant. A fungus capable of agglutinating red cells after growth in normal serum was obtained in only two of some forty sera that were cultured on Sabouraud's medium but positive cultures were not obtained in 20 cases.

The factor was not sedimented by a gravitational field of 50,000–90,000 for one hour which might mean that it was well below 50 mμ in size. It did not dialyze through a collodion sac.

It was not found in fresh sera from cases of infectious hepatitis. One of 30 sera from 10 cases of atypical pneumonia was able to agglutinate chicken red cells in a dilution of 1/200. Of sixteen old sera from 9 cases of non-infectious jaundice, only one was positive, this one grossly contaminated with bacteria. On the basis of the above evidence it is possible that this agglutination was caused by the virus of infectious hepatitis which had been unmasked by long storage and/or bacterial growth, but it is more likely that bacterial contaminants or persisting metabolic products of bacteria are responsible.

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INFLUENCE OF SEX UPON THE LETHAL EFFECTS OF AN HEPATOTOXIC ALKALOID, MONOCROTALINE*

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In human beings, Laennec's cirrhosis is more than twice as frequent in males as in females (1). This fact has usually been attributed to the greater incidence of alcoholism in males. However, the incidence of cirrhosis is also greater in male adults who profess to be non-alcoholics (2). This suggests that the male sex hormones may in some manner increase the susceptibility of human beings to chronic hepatic damage. No experimental evidence has been published to support this view. On the other hand, the effect of estrogens on liver damage in experimental animals has been studied by György and his associates. They reported that female rats were less susceptible than males to the toxic effects of inhalation of carbon tetrachloride (3). Furthermore, estrone partially protected rats against dietetic cirrhosis (4), and particularly when administered in combination with methionine, exerted a striking lipotropic effect (5). These authors reviewed earlier studies which had shown that female rats were less susceptible than males to deficiency of cystine (6) or choline (7), and that female mice were less susceptible than males to the nephrotoxic effects of chloroform (8, 9).

The susceptibility of rats and mice to the effects of chlorinated hydrocarbons and of dietetic deficiency, then, appeared to be influenced by the animal's sex. During a study of the influence of diet upon the lethal effects of monocrotaline, an hepatotoxic alkaloid, it was noted by chance that male rats fed certain of the diets died more quickly than females (10). Because of irrelevant circumstances, these diets, although all qualitatively similar varied somewhat quantitatively in a manner to be described. This fortuitous observation suggested the use of monocrotaline as a tool for the analysis of the relation between sex and the susceptibility of rats to hepatotoxic agents.

* These studies were conducted under contract with the Office of Naval Research, U. S. Navy.

Monocrotaline was first isolated by Neal and his associates (11) from *crotalaria spectabilis*, a legume used to enrich the soil. *Crotalaria spectabilis* is toxic for cattle (12), swine (13), chickens (14, 15), quail (15), doves (15), horses (16), and goats (16). In general, the administration of *crotalaria* resulted in severe hemorrhagic congestion of the liver with necrosis of those liver cells nearest the central veins. In addition, congestion of the lungs and necrosis of lymphoid tissues were observed (13). This pathologic picture is remarkably similar to that produced by the ingestion of plants of another genus, *senecio* (ragwort or Stinking Willie), a weed responsible for hepatic damage in cattle (17–21), horses (20, 21), rats (22), guinea pigs (22), mice (23), and human beings (22). The similarity between the symptoms of *crotalaria* and *senecio* poisoning apparently has its basis in the chemical nature of their respective alkaloids. Adams and Rogers (24) demonstrated that monocrotaline, derived from *crotalaria*, and alkaloids derived from *senecio* contain, in common, the alkanolamine retronecine.

Harris, Anderson and Chen (25) observed that monocrotaline was toxic for mice and rats, and that the lesions produced were similar to those which followed the administration of *crotalaria*. The experiments to be described indicate that the susceptibility of rats to monocrotaline is influenced by their age and by hormonal and dietetic factors.

METHODS

Hooded rats of the MacCollum hybrid strain were weaned at 21 days and treated as described below. In one experiment, hooded rats of the Harvard strain were used.

Diet.—The rats were allowed tap water as desired, and fed one of the following diets:

Diet A: Ralston Purina Dog Chow as desired. This diet consists of meat meal, dried skimmed milk, wheat germ, dried beet pulp, corn grits, cereal feed from corn and wheat, dried figs, soy bean oil meal, molasses, riboflavin supplement, brewer's dried yeast, Vitamin A and D feeding oils, one per cent steamed bone meal, and one per cent iodized salt. According to the manufacturer, an average sample contained 23.0 per cent protein, 6.0 per cent fat, 9.6 per cent ash, and 6.0 per cent crude fiber. In addition, an average sample contained carotene, 9 mg., thiamin 5.6 mg., and riboflavin 5.0 mg. per 100 grams.

An excess of food was offered each animal; the amount of food consumed daily was not measured.

Diet B (26): Vitamin-test casein (General Biochemicals, Inc.), 4 per cent; dried brewer's yeast, U.S.P. (Fleischman's Type 90B), 16 per cent; powdered corn starch (Argo), 71 per cent; cod liver oil (Peder Devold Company "High Potency"), 1 per cent; peanut oil (Somold, Suffolk Oil Mill, Suffolk, Virginia), 5 per cent; and salt mixture (27), 3 per cent. In addition the diet contained 250 μgm. of thiamine, 312 μgm. of riboflavin, 250 μgm. of pyridoxine, and 1.5 mg. of calcium pantothenate in each 100 grams. The ration contained 12 per cent protein and 6.8

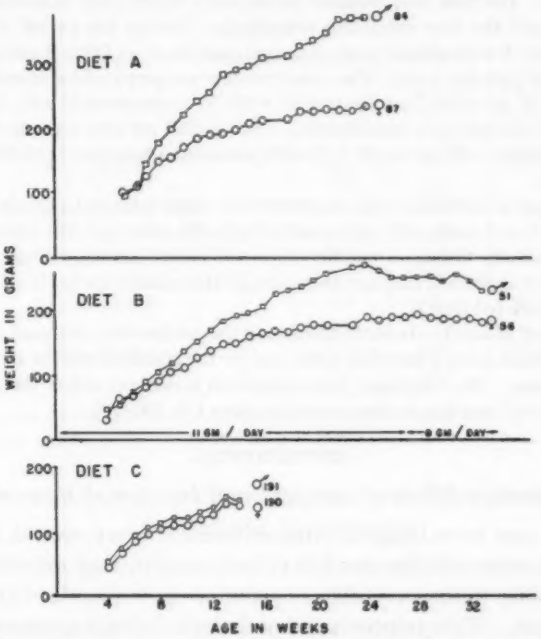

FIG. 1. REPRESENTATIVE GROWTH CURVES OF RATS FED FROM WEANING ON DIETS A, B, AND C

per cent fat. The diet was prepared by mixing the oils and casein together, and then adding a mixture of the other dry ingredients. Food for approximately one week was made at one time, and then stored in the refrigerator at 4°C. The animals were fed four times a week. Each was fed a total of 77 grams of diet per week for 8 to 24 weeks after weaning, and then 56 grams per week for 2 to 6 weeks before the first injection of monocrotaline.

Diet C: Diet B, 56 grams per week after weaning for the entire experimental period.

Typical growth curves have been reproduced in Fig. 1.

Housing.—The rats were individually housed. Those on Diet A were kept in cages with wire mesh bottoms. Those on Diets B and C were housed in cages with removable solid bottoms so that food could not be lost through the bottom of the cage.

Administration of Monocrotaline.—In most experiments, after the preliminary period, rats were injected intraperitoneally every second day with 46 mg. of monocrotaline[1] per kilo of body weight. This is half the reported intravenous LD_{50} dose (25). The rats were weighed immediately before each injection of monocrotaline and the dose calculated accordingly. During the period of injection, rats on Diet A were allowed food as desired, and those on Diets B and C were fed 56 grams of food per week. The monocrotaline was prepared for injection by dissolving it in an equivalent amount of N/14 hydrochloric acid and diluting the solution to one per cent monocrotaline with an 0.85 per cent aqueous solution of sodium chloride buffered to pH 7.35 with potassium phosphate in M/20 final concentration.

Technique of Castration.—In experiments in which castrated animals were used, male rats 3 to 5 weeks old were anesthetized with ether and the testes removed transperitoneally through a mid-line lower abdominal incision. Until these male animals were castrated, they and their female litter mates were fed Diet A. Thereafter, all were fed Diet C.

Statistical Methods.—In each experiment the results were analyzed by the chi-square method, using a four-fold table, and by the standard error of the difference in two means. No differences were considered significant unless the probability that the result was due to chance was less than 1 in 100 (28).

EXPERIMENTAL

1. *Pathologic Effects of Intraperitoneal Injection of Monocrotaline*

When rats were injected intraperitoneally every second day with 46 mg. of monocrotaline per kilo of body weight, they differed in their susceptibility to monocrotaline in a manner to be described in succeeding sections. The symptoms and pathologic findings appeared to vary only with the time of death rather than with age, sex, or dietetic history. The principal pathologic changes observed were necrosis of the hepatic parenchyma and renal tubular epithelium. These changes were more extensive in those rats which died within about 10 days. In those which died later, the damage in the liver and kidneys was definite but less severe. These rats had pulmonary congestion and edema, and dilation of the right side of the heart. The presence of ascites, jaun-

[1] Monocrotaline was obtained through the courtesy of Dr. Roger Adams and Dr. K. K. Chen.

dice and biliuria confirmed the histologic observation that hepatic damage was impressive in both the acute and subacute phases. The lymphoid tissues in the spleen, thymus, and lymph nodes were congested with erythrocytes and the lymphocytes had largely disappeared. Relatively little variation in the pathologic picture was noted from animal to animal.

TABLE I

*Survival Time of Rats Injected with Monocrotaline**

DIET	STRAIN	SEX	NO. OF RATS	AVERAGE WEIGHT**	AVERAGE SURVIVAL TIME	
				gm.	days	
A	MacCollum	♂	21	246	21.7	
		♀	18	155		18.8
B	Harvard	♂	7	234	9.0	
		♀	4	186		18.7
	MacCollum	♂	11	209	9.3	
		♀	7	169		19.3
	Total group B	♂	18	219	9.2	
		♀	11	175		19.1
C	MacCollum	♂	9	143	18.3	
		♀	15	136		18.6

* 46 mg. per kilo of body weight intraperitoneally every second day.

** Average at time of first monocrotaline injection.

2. *The Influence of Age, Diet, and Sex on Survival Time of Rats Injected with Monocrotaline*

One-half the reported intravenous LD_{50} dose (25) of monocrotaline, 46 mg. per kilo, was injected every other day intraperitoneally into rats of both sexes fed various diets. The results of these experiments are shown in Table I and Figs. 2 and 3.

Female rats fed Diet A (Purina Dog Chow) *ad libitum* were, on the average, slightly more susceptible than males to the lethal effects of monocrotaline. However, this difference in susceptibility between the 2 sexes was not statistically significant as tested by the standard error of the difference in the means. Male rats fed this liberal diet were sexually mature and considerably heavier than females. The increased susceptibility of female rats was most apparent in the younger animals (Fig. 3). Among these rats, 8 to 12 weeks old at the time of

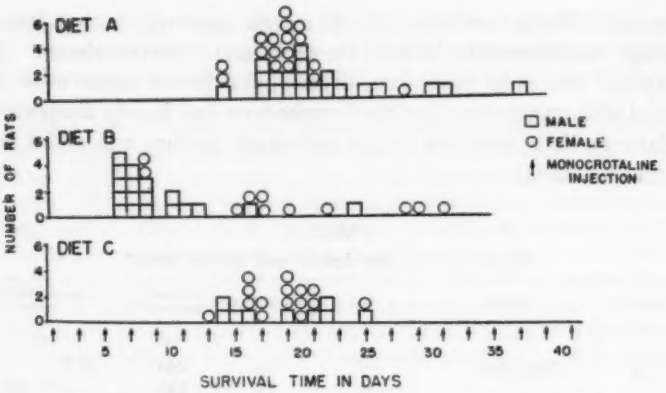

FIG. 2. Influence of Diets A, B, and C and of sex upon the survival time of rats injected intraperitoneally every second day with 46 mg. of monocrotaline per kilo of body weight. In this and the succeeding figures, each block or circle represents an animal. The survival time after the first injection is indicated in days.

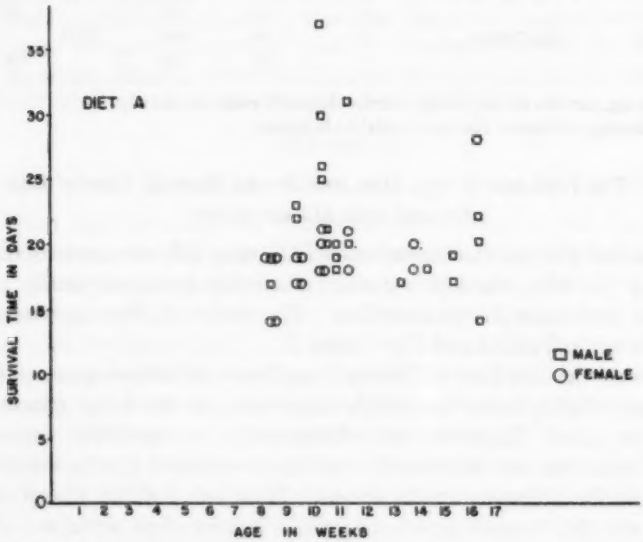

FIG. 3. Influence of age upon the survival time of rats on Diet A injected intraperitoneally every second day with 46 mg. of monocrotaline per kilo of body weight.

the first injection of monocrotaline, all of 15 female rats died within 21 days, at which time 7 of 14 male rats were still alive. This increased susceptibility of young female rats was statistically significant, as tested by the chi-square method.

When rats were fed from weaning a restricted amount of a diet relatively low in protein (Diet C), both sexes grew slowly and at approximately the same rate. When such rats were injected with half the LD_{50} dose of monocrotaline, 46 mg. per kilo, the survival time was found to be equal in the two sexes, an average of 18.2 days, or approximately the same as that of well-fed normal rats. These rats were 9 to 15 weeks old at the time of the first injection of monocrotaline; the age of the rats did not seem to influence susceptibility.

Some rats, in addition to those already described, were placed at weaning on Diet B, which was moderately deficient. On Diet B the rats grew less than on the complete Diet A. However, in contrast to those on the more deficient Diet C, there was some weight difference between the two sexes. The male rats on this diet weighed an average of 220 grams, and the females 175 grams at the time of the first injection, at which time they were 16 to 34 weeks old. When these rats were injected with half the LD_{50} dose of monocrotaline, 46 mg. per kilo, a striking difference in survival time of the two sexes was noted. The female rats like well-fed males and females, and like deficient males and females survived, on the average, 19.1 days. However, male rats fed this semi-deficient Diet B survived on the average only 9.2 days. The susceptibility of the rats in this group did not seem to vary with their ages within the limits studied.

3. *The Effect of Weight and Testosterone Proprionate on the Survival Time of Rats Injected with Monocrotaline*

At least two possible explanations for the greater susceptibility of male compared with female rats fed Diet B must be considered. Perhaps the greater susceptibility of the males was primarily related to their greater size. Or the greater susceptibility may have been due to some other effect of maleness.

The survival times of rats fed the three different diets and injected with monocrotaline were plotted against their weights at the time of the first injection as shown in Fig. 4. It seems unlikely that differences

in weight *per se* can explain the differences in susceptibility between the sexes in rats fed Diet B. These weight differences were actually much less than in rats fed Diet A where no such sex difference in susceptibility was noted. For this reason, the following experiment was designed to test for some other effect of maleness.

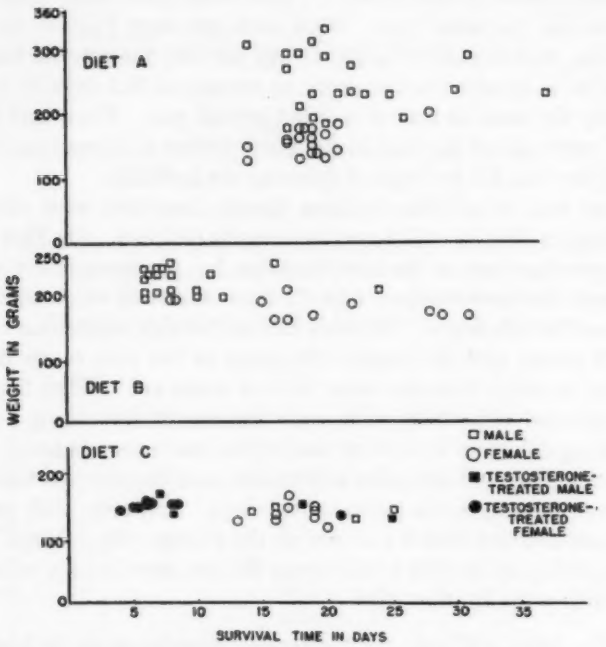

FIG. 4. Influence of body weight and Diets A, B, and C upon the survival time of rats injected intraperitoneally every second day with 46 mg. of monocrotaline per kilo of body weight.

Thirteen male and female rats which had been fed from weaning Diet C for 6 to 8 weeks were injected subcutaneously with 2.5 mg. of testosterone proprionate in sesame oil (Rare Chemicals, Inc.) daily for 2 weeks. Then each was injected intraperitoneally every other day with 46 mg. of monocrotaline per kilo of body weight, while the injections of testosterone proprionate were continued. At the same

time, 13 control rats of both sexes from the same litters and on the same diets were injected with monocrotaline alone. Those animals which were to be injected with testosterone proprionate weighed, on the average, 128 grams before the first injection of testosterone, and the control rats weighed, on the average, 121 grams at this time. Two

TABLE II

*Influence of Testosterone Propionate Upon Survival Time of Rats Injected with Monocrotaline**

GROUP	SEX	NO. OF RATS	WEIGHT**	AVERAGE SURVIVAL TIME
			gm.	*days*
Control	♂	4	139	19.2
	♀	9	137	17.3
Testosterone Proprionate	♂	5	147	12.8
	♀	8	149	7.9

All rats were fed Diet C.

* 46 mg. per kilo of body weight intraperitoneally every second day.

** Average at time of first monocrotaline injection.

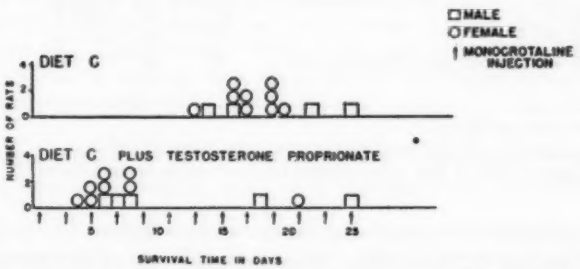

FIG. 5. Influence of testosterone proprionate upon survival time of rats on Diet C injected intraperitoneally every second day with 46 mg. of monocrotaline per kilo of body weight. The rats were injected with 2.5 mg. of testosterone proprionate daily for 2 weeks before and during the injection of monocrotaline.

weeks later, at the time of the first injection of monocrotaline, the testosterone treated rats weighed on the average, 148 grams, and the controls, 137 grams. In Table II and Figs. 4 and 5, it will be seen that injection of testosterone proprionate increased the susceptibility to monocrotaline of male and female rats fed a limited diet (Diet C) of purified foodstuffs relatively low in protein. In this experiment,

the testosterone treated rats died, on the average, in 9.8 days, while the control rats survived 18.0 days. Despite the small differences in weight between the testosterone treated and control rats, a dramatic difference was observed in the susceptibility of the animals to the lethal effects of monocrotaline.

4. *The Effect of Castration, of Estradiol Benzoate, and of Methionine on the Survival Time of Rats Injected with Monocrotaline*

It has been shown in the preceding section, then, that under conditions of restricted food intake, *maleness* increased the susceptibility of rats to monocrotaline. The experiments of György and his associates

TABLE III

*Influence of Castration and of Estradiol Benzoate Upon Survival Time of Rats Injected with Monocrotaline**

GROUP	NO. OF RATS	WEIGHT**	AVERAGE SURVIVAL TIME
		gm.	*days*
Untreated ♀	17	116 (91–138)	22.0
Castrated ♂	7	132 (113–150)	20.9
Castrated ♂ Injected with Estradiol benzoate	8	121 (115–124)	20.4

All rats were fed Diet C.

* 46 mg. per kilo of body weight intraperitoneally every second day.

** Average at time of first monocrotaline injection; range in parentheses.

(3–5) indicated that *femaleness* protected rats against injury by diet and by carbon tetrachloride. Therefore, the rôle of estrogens in the resistance of rats to monocrotaline was studied.

Fifteen male rats were fed Purina Dog Chow *ad libitum* (Diet A) after weaning, castrated 2 to 14 days later, and fed Diet C from then on. Starting 19 days after castration, 8 of these rats were injected subcutaneously every other day with 100 μ gm. of estradiol benzoate in sesame oil (Progynon-B, Schering). After 2 more weeks, these 8 rats were injected intraperitoneally every other day with 46 mg. of monocrotaline per kilo of body weight, while the injections of estradiol benzoate were continued. At the same time, the other 7 castrated males and 17 litter-mate females which had been fed the same diets

for the same times were injected with monocrotaline in the same manner.

The survival times of all these rats are recorded in Table III and Fig. 6. It will be seen that castrated male rats survived an average of 20.9 days. This was about the same time as intact males and females fed either the same restricted Diet C, or the complete Diet A.

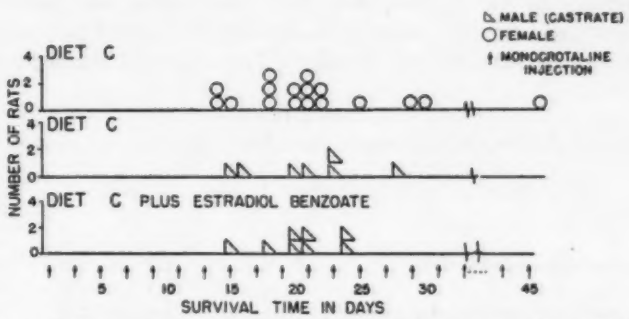

FIG. 6. Influence of castration and of estradiol benzoate upon the survival time of rats on Diet C injected intraperitoneally every second day with 46 mg. of monocrotaline per kilo of body weight. Estradiol benzoate, 100 μ gm. was injected every second day to castrated male rats for 2 weeks before, and during the administration of monocrotaline.

In this experiment injections of estradiol did not appear to prolong the survival time of castrated male rats injected with monocrotaline.

Methionine has been shown to prevent fatty infiltration of the liver in rats fed diets high in fat (30). Furthermore, methionine has been observed to prevent the hepatic necrosis and hepatic cirrhosis produced in rats by diets low in protein (31, 32). And methionine protected protein-depleted dogs against the toxic effects of chloroform (33) and of mapharsen (34). On the other hand, Drill and Loomis (35) were unable to demonstrate that methionine had any beneficial effect upon the hepatic damage produced by carbon tetrachloride in dogs fed either high or low protein diets. Moreover, it has been shown by Earle, Smull, and Victor (36) that large amounts of methionine apparently caused atrophy of liver cells. It seemed of interest to test the effect of methionine on the toxicity of monocrotaline.

The effect of daily subcutaneous injections of 60 mg. of dl-methionine (Merck) in 3 per cent solution was tested in two groups of rats. The

methionine was dissolved in M/20 potassium phosphate aqueous buffer (pH 7.35) containing 0.85 per cent sodium chloride. In the first experiment, the administration of methionine was started simultaneously with the intraperitoneal administration every other day of 46 mg. of monocrotaline per kilo of body weight to rats which had been fed the more restricted Diet C for 9 weeks. In the second experiment, the methionine was injected daily for 7 days before, and during the course of monocrotaline, and simultaneously the control rats were injected with the same volume of buffer. These rats had been fed Purina

TABLE IV

*Influence of Methionine Upon Survival Time of Rats Injected with Monocrotaline**

REGIMEN	NO. OF RATS	SEX	WEIGHT**	AVERAGE SURVIVAL TIME
			gm.	days
A. Control (monocrotaline alone)	2	♂	171 (164–179)	18.5
	3	♀	143 (133–158)	21.7
Methionine and monocrotaline simultaneously	2	♂	164 (157–172)	10.0
	3	♀	144 (133–161)	16.3
B. Control (buffer and monocrotaline)	5	♂	130 (121–142)	13.8
	5	♀	129 (115–140)	20.0
Methionine preceding and simultaneous with monocrotaline	6	♂	135 (125–148)	10.8
	5	♀	129 (98–143)	14.8

All rats were fed Diet C from weaning except 8 rats, in Group B, which were fed Diet A for 12 days, and then Diet C.

* 46 mg. per kilo of body weight intraperitoneally every second day.

** Average at time of first monocrotaline injection; range in parentheses.

Dog Chow (Diet A) for 3 to 12 days after weaning, and then the more restricted Diet C for 6 weeks before the first injection of monocrotaline.

Under the conditions of these experiments, it was not possible to demonstrate that methionine influenced the length of survival of rats injected with monocrotaline. It is of great interest that, compared simultaneous controls, there was considerably less necrosis in the livers of rats injected with methionine, despite its apparent failure to prolong life. It is possible that the administration of methionine in different dosages or by different routes might have influenced the susceptibility of rats to monocrotaline.

In the second experiment with methionine, male rats were more

susceptible than females in both the experimental and control groups. This increased susceptibility occurred only in the 8 rats which had been fed Diet A for 12 days after weaning and Diet C from then on. These rats were equally divided between the experimental and control groups and their inclusion in the experiment did not appear to influence the outcome. No significant difference was noted between the weights of male and female rats in this experiment.

5. *The Effect of Larger, Daily Injections of Monocrotaline in Rats Fed Purina Dog Chow*

Five male and 5 female rats, 17 to 31 weeks old, which had been fed Purina Dog Chow (Diet A) from weaning, were injected intraperi-

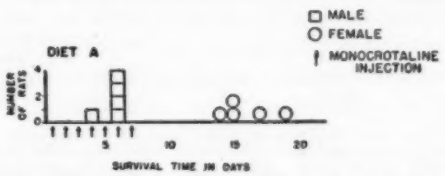

FIG. 7. Delayed lethal effect in rats on Diet A of seven daily intraperitoneal injections of 92 mg. of monocrotaline per kilo of body weight.

toneally daily for 7 days with 92 mg. of monocrotaline per kilo of body weight. This amount is the reported intravenous LD_{50} dose (25). The male rats averaged 341 grams in weight, and the females 199 grams at the time of the first injection. The amount of food consumed was not measured, but it was apparent that the animals ate much less after the first injection than earlier.

One male rat died on the fourth day, and the other 4 on the sixth. At this time, all the females were still alive (Fig. 7). The daily injections of monocrotaline were discontinued after 7 days. The female rats lost weight gradually, and died from 14 to 19 days after the first injection, an average of 16 days later. The gross and microscopic picture in these rats could not be distinguished from other rats which died at this time after injections of 46 mg. of monocrotaline per kilogram of body weight every second day. Thus it appeared that the same sex differences previously noted in moderately deficient rats injected with monocrotaline were apparent in initially well-nourished

animals injected with larger, more frequent doses of this alkaloid. Moreover, a delayed lethal effect was noted in rats in which the administration of the alkaloid had been discontinued.

6. *The Delayed Effect of Relatively Small Doses of Monocrotaline*

Twelve male and 6 female rats were fed the more restricted moderately low protein diet (Diet C) for 12 to 14 weeks and then given 5 injections of 23 mg. of monocrotaline per kilo of body weight at twice-weekly intervals. During the first few days succeeding the last injection of monocrotaline several of the rats failed to finish their daily diet of 8 grams. During this period, the amount of food offered each of the other rats was curtailed to equal the amount eaten by these animals. Sixteen days after the first injection, 2 male rats died. All

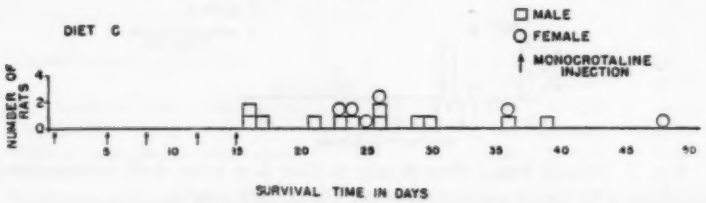

FIG. 8. Delayed lethal effect in rats on Diet C of 5 twice-weekly intraperitoneal injections of 23 mg. of monocrotaline per kilo of body weight.

the remaining animals died at intervals thereafter, the last 48 days after the first injection. On the average, the male rats survived 25.2 and the females 20.3 days after the first injection of monocrotaline (Fig. 8). This difference was not statistically significant.

The pathologic picture observed in these rats which died some time after the last injection of monocrotaline did not differ from that of rats which died at the same time after injection every second day with 46 mg. of the alkaloid per kilo of body weight. As in the previous experiment, death occurred at a considerable interval after the administration of monocrotaline had been discontinued. It is of note that with this much smaller dosage of alkaloid the average survival time of these rats was only moderately prolonged compared with rats given 46 mg. of monocrotaline per kilo of body weight every second day.

DISCUSSION

The experiments described demonstrated that the susceptibility of rats to the lethal effects of monocrotaline was influenced by their age and sex, the particular diet they ate, and the dosage of the alkaloid injected. Variations in any of these factors changed the relative susceptibility of the animals. Under certain conditions of age, diet, and dosage, male rats were more susceptible than females to the lethal effects of monocrotaline. This increased susceptibility could be induced in female rats by the administration of testosterone proprionate. Castrated male rats, however, were as resistant as females, and the administration of estradiol benzoate to these castrated rats did not afford increased protection.

The mechanism by which *maleness* or the administration of testosterone proprionate increased the susceptibility of rats to monocrotaline is not clear. We have made no studies to observe whether an anatomic basis exists for the difference in the susceptibility of the two sexes. This has been suggested to explain the greater susceptibility of male mice to chloroform (9, 37). However, the male rats' nutritional status was of the greatest importance in determining the duration of survival. The sexual difference in susceptibility was evident only in those animals which were first fed a diet such that male rats grew larger than female, and then were fed, before the administration of monocrotaline, a more deficient diet (Sections 2 and 4). Or, in one experiment with well nourished rats, sufficient monocrotaline was administered to produce anorexia and consequently an acute deficiency state (Section 5).

Either acute or chronic dietetic restriction, then, in sexually differentiated rats, increased the susceptibility of the male to the lethal effects of an hepatotoxic alkaloid. Since fully grown male rats are larger than females, one would expect that when both sexes are fed equal, restricted amounts, the males would have relatively less food available than the females. The greater size of the males, however, was not the only determining factor in susceptibility. Both male and female rats injected with testosterone proprionate were more susceptible than control rats of the same weight which were fed the same quantities of the same diet. This was true even though relatively large

changes in the dosage of monocrotaline effected relatively small changes in the survival time (Section 6).

The increased susceptibility of deficient male or testosterone treated rats, therefore, did not seem related necessarily to the greater size of the males. Perhaps under the conditions of these experiments, the composition of the various tissues was modified by the androgens. Biochemical differences between male and female rats have been noted (38–43), and the androgens have been reported to have metabolic activities not necessarily related to their primary sexual effects (44–47). Conceivably, under the influence of the androgens, nutrients needed for protection against monocrotaline were diverted to other uses. This diversion of nutrients may explain the greater susceptibility of male rats, or rats injected with testosterone proprionate, compared with females, to the lethal effects of monocrotaline. However, if a surplus of food were provided, sufficient amounts of the essential nutrients would be available for both purposes.

When the conditions of the experiment were changed, the relative susceptibility of male and female rats was also changed. For example, age seemed an important factor. Among young rats, 8 to 12 weeks old, fed Purina Dog Chow (Diet A), females were more susceptible than males to the effects of injections of monocrotaline, a difference not observed in older rats on this diet. This was the reverse of what was found in rats fed a relatively low protein diet (Diet B) or injected with testosterone proprionate.

In certain experiments, the rats died 1 to 23 days after the last injection of monocrotaline. This same phenomen of death after a latent period has been described repeatedly in the literature in livestock which have eaten *crotalaria*. This suggests either that significant amounts of monocrotaline, or an active fraction, are retained in the body; or that it initiates irreversible changes in the animal's metabolism.

The experiments reported emphasize the important influence of age, sex, and diet upon the susceptibility of rats to hepatotoxins. Undoubtedly, other factors, not specifically studied, also influence susceptibility. The data reported here emphasize the importance of relatively small changes in the design of an experiment in determining its outcome.

SUMMARY

(1) The influence of age, sex, and diet upon the lethal effects of monocrotaline, an alkaloid derived from *crotalaria*, has been reported. Under the conditions studied, the administration of monocrotaline to rats resulted in necrosis of the liver and renal tubules, congestion of the lymphoid tissue with erythrocytes, and, in rats which died after about ten days, pulmonary congestion and edema.

(2) In general, no difference was noted in the survival time of male and female rats fed a supposedly normal diet and injected with monocrotaline. However, if only rats 8 to 12 weeks old were considered, females survived a shorter time than males.

(3) Male rats, fed a relatively low protein diet, died in approximately half the time of females fed the same diet or males and females fed the normal diet.

(4) Rats of both sexes fed a still more deficient diet were as resistant as normal rats.

(5) The administration of testosterone proprionate increased the toxicity of monocrotaline for both sexes on the more deficient diet. This was true even though testosterone-treated and control rats weighed approximately the same amount. Neither castration nor the administration of estradiol benzoate influenced the survival time of rats on this latter diet.

(6) The experiments suggest that the greater susceptibility of deficient male rats to monocrotaline may be due to the metabolic effects of male sex hormones.

We wish to acknowledge the valuable technical assistance of Mrs. Betty Jean Stetson and Miss Aileen Mulvey.

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ATTEMPTED HETEROLOGOUS TRANSPLANTATION OF CARCINOMA OF THE CERVIX

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Greene (1, 2, 3) reported several instances of successful heterologous transplantation of malignant tumors into the anterior chamber of the guinea pig's eye using human carcinoma and sarcoma tissue. Hovenanian and Deming (4) obtained growth in the guinea pig's eye using human prostatic carcinoma which had metastasized to the inguinal area. Greene felt that this biologic test demonstrated autonomy of the malignant tissue in a more exact manner than obtainable by clinical and microscopic studies. Autonomy of malignant tissue is present when this tissue can invade and live in a foreign tissue, a tissue other than the parent of the neoplasm. Using this biologic concept of malignancy and autonomous growth, Greene would question the usual microscopic diagnosis of malignancy based upon anaplastic cellular changes and evidence of local tissue invasion or lymph vessel and lymph node spread.

To discount the microscope, to alter established microscopic evidence, and to accept heterologous transplantation as the only method to determine autonomous growth goes against the teachings and beliefs of most pathologists. It makes the diagnosis entirely too rigid and would delay rather than enhance the all important efforts at early diagnosis and treatment of cancer. As a method for studying malignant tissue, it has appealing advantages.

Carcinoma of the cervix presents two particular problems which successful intraocular heterologous transplantation might assist in solving. It has been recommended that epidermoid carcinoma of the cervix be divided into spinal, transitional, and basal types according to the preponderant microscopic cell type. The unconfirmed, but general impression exists than spinal cell carcinoma of the cervix is less radiosensitive and less rapid in its spread and basal cell carcinoma more radiosensitive and more rapid in its clinical advance. Since repeated

cervical biopsies or even a single biopsy in separate areas will show an entirely different type of cell, most of the cases have been lumped into the middle category as transitional. Greene has shown that the tissue of origin becomes more evident by heterologous transplant study, and it was hoped, that if such a method were successful using human cervical carcinoma, the preponderant epidermoid cell type could be determined. The second problem deals with the earlier stages of cervical malignancy, the intraepithelial, pre-invasive, or carcinoma-in-situ stage about which there has been so much discussion. Galvin and TeLinde (5) felt that this represented an early stage of true carcinoma and their large series of cases suggested this conclusion from a microscopic angle. Although Greene termed this type of lesion precancerous, he found similar lesions of unstated origin incapable of growth in anterior chamber transplant experiments. A successful biologic test would do much to clarify this microscopic interpretation.

MATERIAL AND METHOD

Twelve patients with carcinoma of the cervix which had advanced beyond the confines of the cervix were used. Three cases were classified as League of Nations II, seven as League of Nations III, and two as League of Nations IV. None had begun treatment and cases which by examination and biopsy showed extensive necrosis or overwhelming infection were not used. All of the above biopsies showed a heavy epithelial component and a not unusual amount of desmoplastic reaction.

Biopsies of the carcinoma were taken in as papillary an area as possible and these were placed in a sterile petri dish containing distilled water with 500 to 1,000 units of penicillin per cc. Transplants were done within three hours after the biopsy was secured.

Eight adult female guinea pigs were used for each biopsy. Using sterile precautions the eye was locally anesthetized with pontocain solution and the anterior chamber was entered by means of a corneal knife incision at the corneoscleral junction. Through this incision a trocar containing tissue with about a 0.5 mm. diameter was inserted and the tissue guided to the lower iris angle by a blunt instrument pressed externally.

RESULTS

A foreign body reaction associated with some infection was a prominent feature during the first six to ten days of observation in all cases. The eyes of all animals in one case and two to six animals in most of the cases never recovered from the infection; the transplant hyalinized eventually and the eye became completely clouded. In all but one

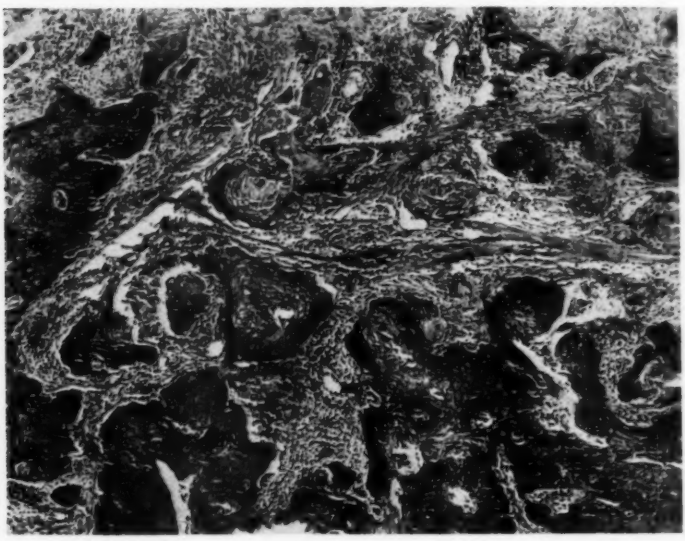

FIG. 1. Epidermoid carcinoma of the cervix, spinal cell type, in a biopsy taken at the same time that tissue was obtained for anterior chamber transplantation. Note the pearl formation.

case two to four of the eyes in each group of eight guinea pigs showed an apparently healthy transplant with beginning vascularization of the tissue by twelve to fourteen days. At no time was there definitely discernible growth and by nineteen to twenty-eight days the apparent "takes" had become smaller and almost a dead white in color. The animals have been observed from six weeks to fourteen months without evidence of delayed or slow growth.

In several instances the eye was sacrificed for microscopic study

after it was evident that there was a diminution in size and devascularization. Only two eyes in two separate cases were found to contain viable malignant tissue, twenty-two and fifteen days respectively after the original transplantation. Both fragments were quite small. One case was classified as a spinal cell type of epidermoid carcinoma by biopsy (Fig. 1) and the transplant did show the formation of a pearl

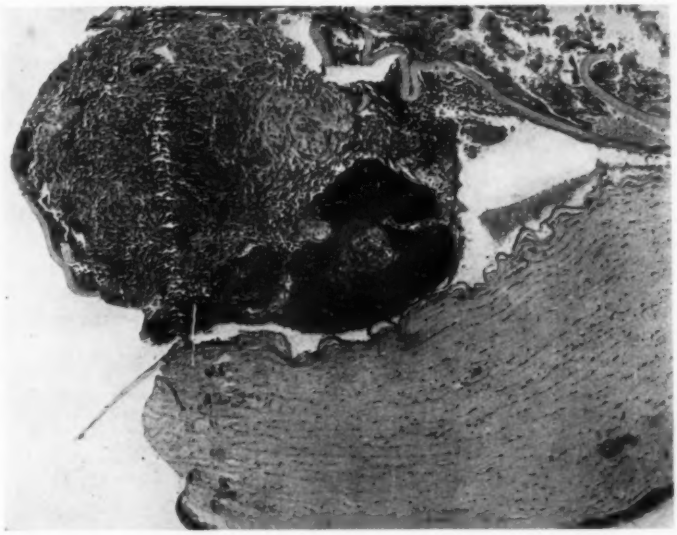

FIG. 2. Carcinoma with pearl foundation still viable in the anterior chamber of guinea pig's eye twenty-two days after transplantation of the type of tissue shown in Fig. 1.

twenty-two days later (Fig. 2). The other case was transitional by biopsy (Fig. 3) and fifteen days later the transplant was a similar cell type (Fig. 4). Microscopic studies of other guinea pigs' eyes six weeks to several months after attempted transplant revealed no malignant tissue. A few serial transplants were attempted from fourteen to twenty-one days after the original transplant, but none of these even gave a suggestive "take." The lack of primary growth made such serial transplanting impractical.

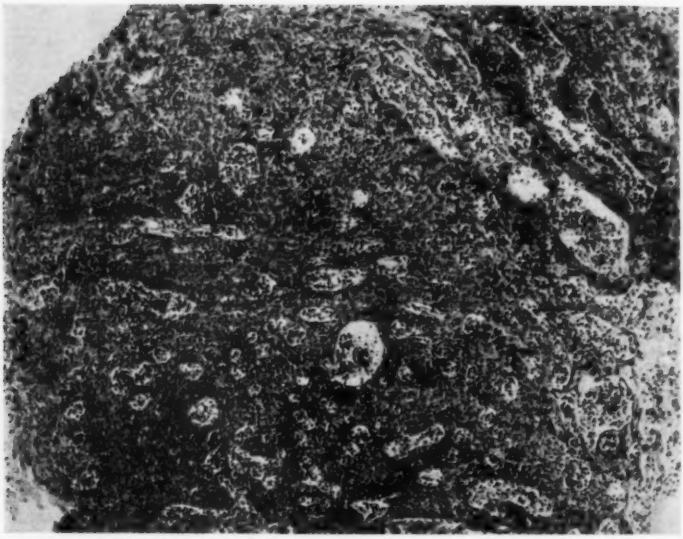

FIG. 3. Epidermoid carcinoma of the cervix, transitional cell type, in a biopsy taken at the time of securing tissue for heterologous transplantation.

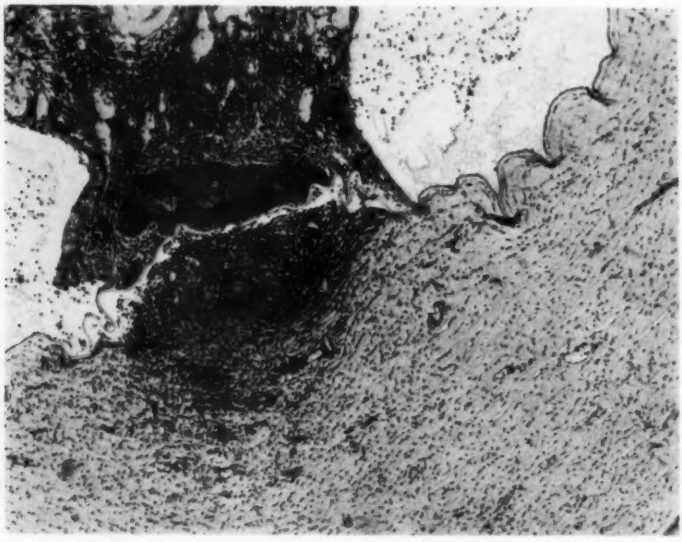

FIG. 4. Microscopic section of a guinea pig's eye fifteen days after transplantation of human cervical carcinoma of the same type and from the same case as shown in Fig. 3.

SUMMARY AND CONCLUSION

Heterologous transplantation of human carcinoma of the cervix into the anterior chamber of a guinea pig's eye was attempted. It was hoped that this would demonstrate a preponderant epidermoid cell type (spinal, transitional, or basal) more accurately than the microscope. If these transplants were successful, the experiment could be extended to the intraepithelial carcinoma or carcinoma-in-situ group of cervical epithelial changes.

Twelve untrated and relatively late or far-advanced cervical carcinomas were selected. Care was used to obtain tissue as rich in epithelial cells as possible and with a minimum of infection, necrosis, and fibrotic response. None of the transplants were successful, but in two instances tiny islands of viable malignant tissue were found in the guinea pig's eye fifteen and twenty-two days respectively after the original transplant. One of these cases, a spinal cell type by original biopsy, generated a pearl in the transplant.

It is concluded that the heterologous transplantation technique for the study of human malignant tissue is not applicable to human carcinoma of the cervix. The unavoidable associated secondary infection of the cervical neoplastic tissue and the variable, but ever-present fibrous scarring are probably responsible for the failure of the method.

Note: This study was made possible by a grant from Mr. and Mrs. George W. Frederick, Baltimore, Maryland.

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OBSERVATIONS ON THE EFFECTS OF TETRAETHYL PYROPHOSPHATE (TEPP) IN MAN, AND ON ITS USE IN THE TREATMENT OF MYASTHENIA GRAVIS[1]

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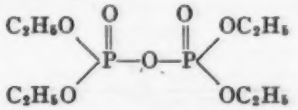
Several esters of phosphoric acid are potent inhibitors of the cholinesterase (ChE) enzymes and as a result have important pharmacological properties. One of these esters, di-isopropyl fluorophosphate (DFP), has been shown by Adrian and his co-workers (1) to inhibit ChE irreversibly. The administration of this compoiund to human subjects resulted in characteristic effects attributable to the depression of ChE activity in the tissues (2–5). In addition to the usual muscarinic and nicotinic effects of cholinergic drugs, central nervous system symptoms were prominent following the administration of DFP, and were accompanied by easily recognizable electroencephalographic changes. The effect of DFP on neuromuscular function, however, was not as striking following intravenous or intramuscular administration to either normal or myasthenic subjects as one might expect from its potent action in inhibiting ChE. In contrast, when DFP was administered by direct arterial puncture, the nicotinic effects in the muscles supplied by the injected artery were very striking and prolonged. This difference, and the development of central nervous system symptoms following the administration of DFP, were in contrast to the effects of neostigmine administration. This suggested that the solubility properties of these drugs might be important in determining the pattern of their physiological effects, as had been proposed for various neostigmine analogues by Schweitzer, Stedman and Wright (6). As a result, a search was made for other phosphoric acid derivatives which were more highly soluble in water and at the same time retained the ability to inhibit ChE strongly. It was postulated that such substances would have a much more marked effect on neuromuscular conduction than DFP, with less disturbance of central nervous system

[1] The work described in this paper was carried out under a contract between the Medical Division, Chemical Corps, U. S. Army, and the Johns Hopkins University.

function, and might be valuable agents in the treatment of myasthenia gravis (5).

Tetraethyl pyrophosphate (TEPP) was thought worthy of investigation from this point of view and has been under study in this laboratory for the past year. A brief communication has already been made outlining the results (7). At the same time studies with this drug have been carried out in England and have been the subject of preliminary reports (8, 9).

TEPP was prepared as early as 1854 (10) and its chemical structure is believed to be the following (11):



During the war the Germans introduced this substance as an insecticide and it is now used for this purpose (12). TEPP, and the related hexaethyl tetraphosphate (HETP), were found to be potent anticholinesterase agents, with more activity than DFP against rat ChE both in vivo and in vitro (13, 14). When administered to mammals they produced muscarinic, nicotinic and central nervous system effects which resembled those seen following DFP (8, 15).

This communication will describe the results of the administration of TEPP to 18 normal human subjects and to 12 patients with myasthenia gravis. Observations will be reported on the effect of TEPP on ChE enzymes in vitro and in vivo, the general systemic effects of TEPP, and its effect on the gastro-intestinal tract, the central nervous system, and the peripheral neuromuscular unit.[2] Also, the results of its use in the treatment of 12 patients with myasthenia gravis will be discussed.

The Effect of TEPP on the Cholinesterase Activity of Plasma, Red Blood Cells, Muscle and Brain in Vitro

Human plasma ChE was more sensitive to the inhibitory effect of TEPP than the cholinesterases present in muscle, brain, and red blood cells, which were less, and approximately equally sensitive (Fig. 1).

[2] The method of making ChE determinations and the technics for the various physiological studies have been outlined in detail in the studies previously reported on DFP (2–5).

The relation between the concentration of TEPP and the degree of inhibition of ChE activity was linear except for concentrations that caused over 90 per cent inhibition. TEPP was a more potent inhibi-

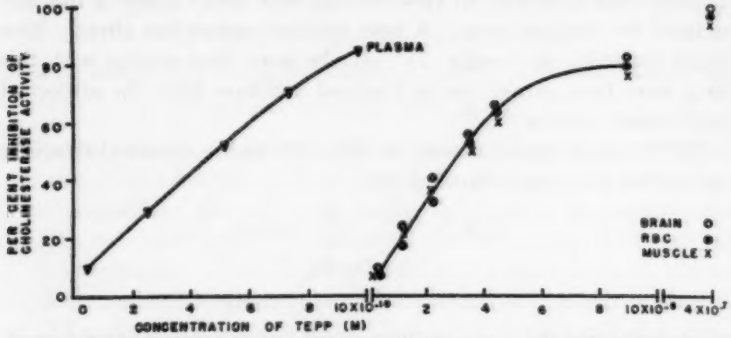


FIG. 1. The inhibition of plasma, brain, red blood cell, and muscle cholinesterase activity by TEPP in vitro. The concentrations of the enzyme preparations are as recorded in Table I.

TABLE 1

The in vitro Sensitivity of Human Plasma, Red Blood Cell, Brain, and Muscle Cholinesterases to Inhibition by TEPP, DFP, and Neostigmine

The molar concentration of each anticholinesterase agent is recorded which produced 50 per cent inhibition of the ChE activity of a solution or homogenate which hydrolyzed 4.78×10^{-4} mM acetylcholine bromide per hour in the absence of inhibitor. This rate of hydrolysis was produced by 51 mg./ml. of plasma, 124 mg./ml. of brain (cerebral cortex) homogenate, 58 mg./ml. of muscle (psoas) homogenate, and 34 mg./ml. of red blood cell contents. The initial concentration of acetylcholine bromide was 0.015 M. ChE and anti-ChE compounds were mixed 45 minutes before the addition of acetylcholine.

	PLASMA ChE	BRAIN ChE	MUSCLE ChE	RED BLOOD CELL ChE
TEPP	5×10^{-9}	3.2×10^{-8}	3.5×10^{-8}	3.5×10^{-8}
DFP	9.5×10^{-9}	3×10^{-7}	2.5×10^{-7}	4×10^{-7}
Neostigmine	4×10^{-8}	4×10^{-7}	3×10^{-7}	8×10^{-7}

tor of all four enzymes than was DFP or neostigmine (Table 1). All the anticholinesterase agents were more effective against plasma ChE than against the other human ChE enzymes.

The inhibition of any of the above ChE enzymes by TEPP was not

an instantaneous reaction, but occurred over a period of minutes at a rate which was more rapid at higher concentrations of TEPP (Table 2), and at lower concentrations of enzyme. The combination of TEPP and ChE could not be reversed in vitro by dilution at 23°C within the first four hours (Table 2), in contrast to the transient rever-

TABLE 2

Inactivation of Cholinesterase (Red Blood Cell) by TEPP at Varying Periods of Incubation at 23°C, and Relationship to Concentration of TEPP

Acetylcholine and sodium bicarbonate were added for the determination of ChE activity after incubation of TEPP with the red blood cell solution. The final concentrations of acetylcholine and of ChE in the dilution experiments were the same as in Table 1, and in the undiluted series fourteen times as great.

The inactivation of ChE was slower when the concentrations of ChE and TEPP during incubation were lower. Fourteen-fold dilution of ChE and TEPP after incubation did not result in restoration of enzyme activity during the period of observation. Similar results were obtained with plasma, muscle and brain cholinesterases.

TEPP AND ChE	UNDILUTED	DILUTED 14 TIMES	
		After incubation	From the start
TEPP conc. (M) during incubation...	3×10^{-7}	3×10^{-7}	2.17×10^{-8}
TEPP conc. (M) when ChE was determined.....	2.95×10^{-7}	2.1×10^{-8}	2.1×10^{-8}
TIME OF INCUBATION OF TEPP AND ChE	INHIBITION OF ChE ACTIVITY		
minutes	*per cent*	*per cent*	*per cent*
0*	90	8	10
5	95	77	32
10	96	90	51
15	97	90	60
30	95	94	71
45	95	94	90
240	96	96	96

* TEPP added to ChE at the same time as acetylcholine.

sibility of ChE inhibition by DFP, which has been demonstrated over a narrow range of inhibitor concentration during the first hour (16). However, whereas the subsequent inhibition of ChE by DFP is permanent (16, and Table 3), some restoration of ChE activity following inhibition by TEPP was observed after standing for 20 or more hours (Table 3). This restoration of ChE activity was very slight at 5°C,

more marked at 37° than at 23°C, though always incomplete, and somewhat more marked after dialysis, Since the half hydrolysis time of TEPP (in distilled water) is eight hours at 23°C, and four hours at 37° C (17), the time relations suggest the possibility that the slow hydrolysis of any free TEPP might gradually shift the equilibrium in the direction of dissociation of the TEPP-ChE complex. The presence of an enzyme in the tissues capable of accelerating the hydrolysis of TEPP

TABLE 3

The Effect of Prolonged Standing and of Dialysis on the Inhibition of Cholinesterase (Red Blood Cell) by TEPP, DFP, and Neostigmine

Acetylcholine and sodium bicarbonate were added for the determination of ChE activity after the indicated periods of time. The concentrations of acetylcholine and ChE were the same as in Table 1. The presence of toluene (0.25 cc.) completely inhibited bacterial growth.

Following inhibition by TEPP the ChE activity could be partly restored by prolonged standing, especially at 37°C or with dialysis. In contrast, inhibition by DFP was permanent, while inhibition by neostigmine was completely reversed by dialysis. The presence of neostigmine protected the ChE against inhibition by TEPP. Similar results were obtained with brain, muscle, and plasma cholinesterases, except that restoration of activity of the latter following inhibition by TEPP was less.

	CONCENTRATION (M)	PER CENT INHIBITION OF ChE ACTIVITY AFTER STANDING FOR:				
		1 hr. at 23°C	96 hrs. at 5°C	24 hrs. at 23°C	24 hrs. at 37°C	24 hrs. at 23°C (with dialysis)
TEPP	9×10^{-7}	94	94	78	64	60
DFP	7×10^{-6}	92	95	97	88	90
Neostigmine	6×10^{-6}	90	75	90	70	0
Neostigmine and TEPP*	6×10^{-6} / 9×10^{-7}	96	78	95	90	8

* TEPP added 30 minutes after neostigmine.

or the dissociation of the TEPP-ChE complex has not yet been demonstrated, but is suggested by the observation that restoration of ChE activity following depression by TEPP occurs to a somewhat greater extent in red blood cell, brain, and muscle homogenates than in plasma, and considerably more rapidly and more completely in vivo than in vitro.

As had been demonstrated for DFP (18), TEPP was incapable of inactivating ChE which was already in combination with neostigmine. Such a protective action was demonstrated by adding TEPP to ChE

which had been almost completely inactivated by neostigmine. When uncombined TEPP was then removed by hydrolysis and dialysis, and the neostigmine by dialysis, the ChE resumed its original activity. This was in contrast to ChE which had been inactivated by TEPP without prior protection by neostigmine (Table 3).

The Water-Lipoid Partition of TEPP, DFP and Neostigmine

The physiological effects of anticholinesterase agents are determined not only by their potency as inhibitors of specific cholinesterases and by the persistence of this inhibition, but also by their ability to reach these various cholinesterases. One important factor determining this penetration may be their relative solubility in aqueous and lipoid sol-

TABLE 4

The Water-lipoid Partition of TEPP, DFP, and Neostigmine

The per cent of each anticholinesterase agent extracted from aqueous solution by an equal volume of peanut oil is recorded. The concentrations of anticholinesterase agents and of ChE are the same as in Table 1.

	PLASMA ChE	MUSCLE ChE	RED BLOOD CELL ChE	BRAIN ChE
TEPP	0	14	0	9
DFP	84	87	90	85
Neostigmine	11	15	16	20

vents. Compounds which are more soluble in water might be expected to reach muscle ChE in higher concentrations than brain ChE, which may exist in a lipoid phase. Conversely, compounds which are more soluble in lipoid might be expected to reach brain ChE in higher concentration. The water-lipoid partitions of TEPP, DFP, and neostigmine were compared by thoroughly mixing aqueous solutions of these compounds with peanut oil, removing the peanut oil by centrifugation, and determining the anti-ChE activity of the remaining aqueous solution. The concentration of TEPP in the aqueous phase was only slightly reduced by extraction with peanut oil, that of neostigmine a little more, while that of DFP was very markedly reduced (Table 4). The DFP removed from the aqueous phase could be demonstrated quantitatively in the lipoid phase by the effect of the peanut oil in vivo on the ChE activity of plasma and red blood cells.

The Effect of TEPP on Plasma and Red Blood Cell Cholinesterase Activity in Vivo

Studies were made following the administration of this drug to human subjects orally, intramuscularly, intravenously and intra-arterially. For intravascular administration the TEPP was in aqueous solution which was freshly prepared for each experiment, as it hydrolyzes in water within several hours (17). Intramuscular injections were made in aqueous solution, in peanut oil, and in propylene glycol. TEPP is stable for many weeks in the latter two vehicles, which were also used

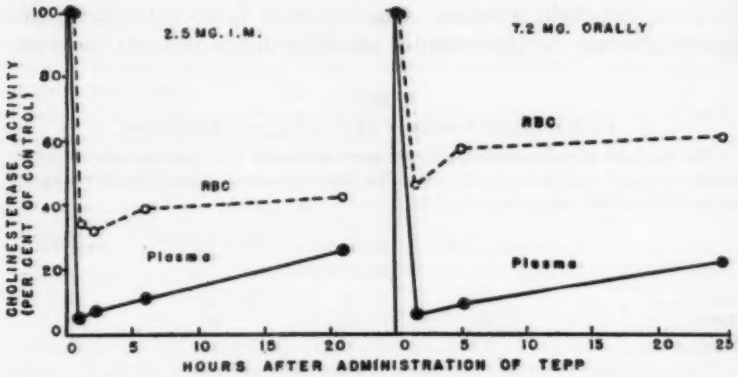


FIG. 2. The effect on plasma and red blood cell cholinesterase activity of a single dose of TEPP administered intramuscularly (in aqueous solution) and orally (in propylene glycol). (Subject G. R.)

for oral administration. The absorption of the drug from the gastrointestinal tract was better when dissolved in propylene glycol. For oral administration a concentration of 5 mg. TEPP per cc. of propylene glycol was employed. This solution was measured into water by means of a calibrated dropper immediately prior to administration.

The intramuscular or intravascular administration of 1 mg. or more of TEPP resulted in a rapid depression of the ChE activity of the plasma to near zero, and of the red cell ChE activity to 60 per cent or less of that originally present (Figs. 2 and 3). Approximately four times as large a dose was required to produce a similar effect when the compound was administered orally. As would be expected from the stud-

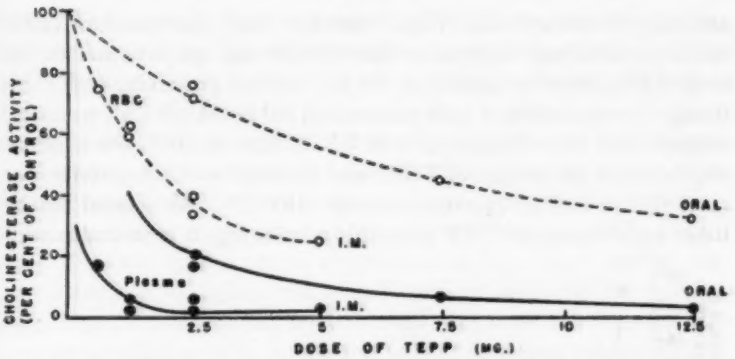


FIG. 3. The effect on plasma and red blood cell cholinesterase activity of graded single doses of TEPP administered intramuscularly (in aqueous solution) and orally (in propylene glycol).

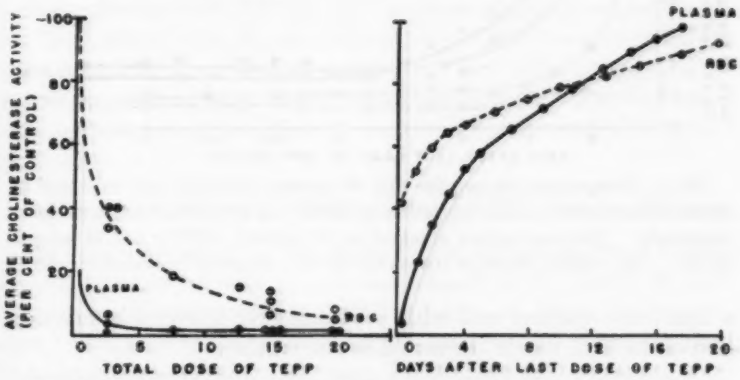


FIG. 4. The effect of the daily intramuscular administration of TEPP (2.5 mg. q.d. in aqueous solution) on plasma and red blood cell cholinesterase activity, and recovery after cessation of TEPP. (Average values obtained in 4 subjects (left) and 14 subjects (right).)

ies in vitro (Fig. 1) TEPP had a more striking affinity for plasma ChE, causing a depression to 40 per cent of its original activity before there was any significant effect on red cell ChE.

Single graded, or successive doses of TEPP administered by any route produced increasing depression of the ChE activity of plasma

and red cells toward zero (Figs. 3 and 4). Each increment of TEPP had a progressively decreasing effect which was proportional to the level of ChE activity existing at the time of that particular dose. Although the depression of both plasma and red blood cell ChE was more marked after the administration of TEPP than of DFP, the relationship between the dosage of TEPP and its effect on ChE activity was quite similar to that encountered with DFP (2). As pointed out by Riker and Wescoe for DFP (19), this relationship is comparable with

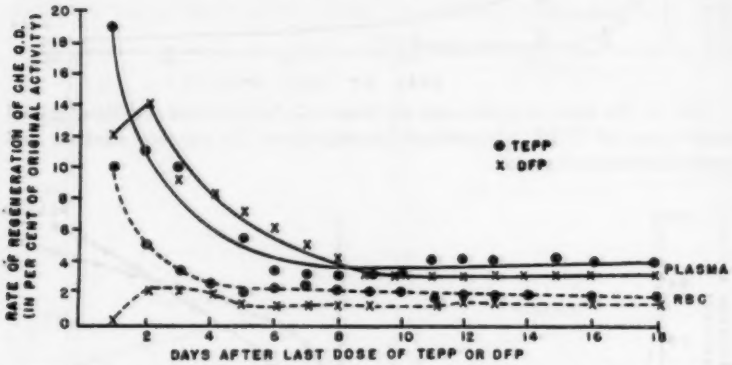


FIG. 5. Comparison between the rate of recovery of plasma and red blood ce cholinesterase activity after depression by TEPP and by DFP administered intramuscularly. (Average values obtained in 14 subjects (TEPP) and 35 subjects (DFP). The vehicle (water or peanut oil) did not influence the rate of recovery.)

a first-order reaction and with predominantly irreversible inactivation of the ChE within the period of observation.

The Rate of Return of Plasma and Red Blood Cell Cholinesterase Activity after Cessation of Administration of TEPP

The maximum depression of plasma ChE activity occurred within one hour after the administration of TEPP by any route (Fig. 2). Following this period there was a gradual return of the plasma ChE activity at an average rate of 19 per cent of the original level during the first 24 hours and 11 per cent during the second 24 hours (Figs. 4 and 5). This rate of return gradually fell off to about 4 per cent per day by the sixth day, and 3 per cent by the tenth day. The rate of return of

plasma ChE activity after depression by TEPP was somewhat more rapid during the first 24 hours than after depression by DFP, but following this period the rates of return were approximately the same (Fig. 5).

The maximum depression of red blood cell ChE activity occurred within two hours after the administration of TEPP by any route. The return of this ChE activity was also most rapid during the first 24 hours, increasing at an average rate of 10 per cent of original activity (Figs. 4 and 5). The rate of return diminished to 5 per cent during the second day and to slightly less than 2 per cent by the fourth day, at which rate it remained until normal levels were reached. This rate of recovery of ChE activity in the red cells after depression by TEPP was significantly more rapid during the first 24 to 48 hours than after the administration of DFP. After this time it was only slightly greater than that seen following DFP (Fig. 5).

As was the case with DFP the rate of return of plasma and red blood cell ChE activity after the administration of TEPP was independent of the dose of drug, of the time over which it was administered, and of the existing ChE activity. Recovery of ChE activity was always complete, and there was no difference found between normal and myasthenic subjects.

Symptoms Following the Administration of TEPP to Human Subjects

The dosage necessary for the production of symptoms due to TEPP administered parenterally (in aqueous solution or in peanut oil) was 5 mg. in one injection, 3.6 mg. daily for 2 days, or 2.4 mg. daily for 3 days. Similar results were obtained following the oral administration (in propylene glycol) of 7.2 mg. every 3 hours for 3 to 5 doses.

The symptoms that followed the administration of TEPP to human subjects qualitatively resembled those which followed DFP, and included most of the muscarinic and nicotinic effects of cholinergic drugs (except for the absence of any significant peripheral vasodilatation or fall in blood pressure), as well as symptoms referable to the central nervous system (Table 5). However, there were certain very striking quantitative differences between the symptoms produced by TEPP and those which followed DFP.

The nicotinic effects of TEPP were much more striking than those

TABLE 5

Signs and Symptoms that Followed the Administration of TEPP to 16 Subjects (4 Non-myasthenic and 12 Myasthenic) in Doses Described Above, Without Atropine, and in Doses Described below for the Treatment of Myasthenia Gravis, with the Simultaneous Administration of Atropine

Effector organ		*Number of subjects*
1. *Muscarinic*		
a) Gastro-intestinal	anorexia, nausea	15
	vomiting	10
	abdominal cramps (mild)	8
	cardiospasm	5
	diarrhea	5
	eructation, "heartburn"	5
b) Sweat glands	increased sweating	13
c) Salivary glands	increased salivation	6
d) Lacrimal glands	increased lacrimation	2
e) Heart	bradycardia	3
	prolongation of PR and QRS intervals	1
f) Peripheral vascular	subjective warmth, "hot flashes"	4
g) Lungs	respiratory difficulty	4
h) Pupils	miosis	3
i) Ciliary body	difficulty of distant vision	3
j) Bladder	frequency (mild)	3
2. *Nicotinic*		
	local fasciculations at site of i.m. injection, in non-myasthenics	4
	generalized fasciculation, in non-myasthenics	1
	myasthenics	9
	increased strength, in myasthenics	12
	weakness (following overdose), in myasthenics	2
	weakness (slight), in non-myasthenics	3
3. *Central Nervous System*		
	giddiness	15
	uneasiness, restlessness, tremulousness	12
	insomnia	9
	headache	8
	paresthesias	6
	excess dreaming, occ. nightmares	6
	EEG change (slow waves)	3
	drowsiness	2
	tremor	2
	mental confusion	2
	emotional lability, with depression	2

of DFP. When TEPP was injected intramuscularly localized fasciculations appeared in normal subjects, and with higher doses there was slight weakness accompanied by generalized muscular fasciculations. These were most marked in the facial and extra-ocular muscles, sometimes causing uncontrollable jerking movements of the eyes and tremor of the eyelids. In myasthenic subjects there was a pronounced increase in strength and fasciculations were frequently noted, especially in those muscles least involved in the myasthenic process.

The muscarinic effects of TEPP, which were the first to appear, included anorexia, nausea, vomiting, and a sense of pressure beneath the lower sternum suggestive of cardiospasm. Abdominal cramps were mild and fleeting, and diarrhea was observed chiefly when neostigmine was administered subsequent to the TEPP. Profuse sweating and excessive salivation were common. Lacrimation, miosis, difficulty in distant vision, bronchoconstriction, urinary frequency and bradycardia occasionally appeared after larger doses of the drug.

The central nervous system symptoms which followed the administration of TEPP consisted chiefly of uneasiness, restlessness, tremulousness, and giddiness. Larger doses of drug sometimes caused headache and paresthesias, and following prolonged administration there was occasionally drowsiness, tremor, mental confusion, and emotional lability, with depression. Insomnia, excessive dreaming, and nightmares, which were common after DFP, were less frequent and less severe following TEPP. The paresthesias accompanied only the larger doses of the drug and were variously described as a feeling of "tingling," "pins and needles," "warm waves", and "electric shocks". These were usually intermittent and generalized, often beginning in the facial region and spreading over the body and limbs. Electroencephalographic changes following TEPP were minimal and much less frequent than after the administration of DFP. Three patients did develop occasional slow waves in their electroencephalograms, occurring at a rate of 3 to 6 per second and similar to the more striking bursts of slow waves seen following DFP.

When symptoms appeared they usually began suddenly about 30 minutes after the last dose of the drug. Muscular fasciculations were the most transient, almost always disappearing within one hour after onset. Vomiting was also of short duration. The remaining musca-

rinic manifestations, as well as the central nervous system symptoms, usually persisted for priods ranging from 1 to 5 hours. After large doses of TEPP nausea and giddiness were present for as long as 24 hours, and it was quite common for patients to feel comfortable while in the recumbent position after the overt symptoms had worn off, only to have a return of giddiness if they sat up or walked about. This was not accompanied by any change in the blood pressure. Increase in strength in the patients with myasthenia gravis persisted for a period of 12 to 72 hours. Local instillation of TEPP in peanut oil in a concentration of 0.5 mg. per ml. resulted in marked miosis lasting for 12 to 48 hours.

After the administration of TEPP symptoms appeared more abruptly, were more explosive in nature, and also disappeared more rapidly than after DFP. Repeated administration of TEPP, like DFP, resulted in an increasing symptomatic effect of each dose, presumably because of the progressive inhibition of ChE. However, the cumulative effect of repeated doses was not nearly as striking as in the case of DFP, where the potentiation of subsequent doses was seen over a much longer period of time. Potentiation of repeated doses of TEPP was striking within several hours of a given dose but diminished rather rapidly during the subsequent few days, whereas the sensitizing effect of a dose of DFP could be demonstrated for a period of 3 to 4 weeks (3). This difference is presumed to be due to a more rapid return of ChE activity in the tissues after depression by TEPP than by DFP.

The ChE activity of the plasma was reduced to nearly zero before the appearance of symptoms due to TEPP. When TEPP was administered over a relatively short period of time, in doses described above, symptoms appeared at red blood cell ChE levels between 13 and 25 per cent of the original activity. This appearance of symptoms at a particular level of red blood cell ChE activity is probably due to the similarity in the sensitivity of the ChE enzymes of the red blood cells, muscle, and brain to TEPP, as demonstrated in vitro. However, as in the case of DFP, there was no constant relation between the presence of symptoms and the degree of depression of red blood cell ChE activity, probably because of the slower rate of restoration of red blood cell ChE, compared to the tissue cholinesterases. The ChE activity of the red cells could be depressed to very low levels (8 per cent) by TEPP without the occurrence of any symptoms by the daily admin-

istration of small doses (0.5 to 1 mg. daily intramuscularly). When symptoms did occur following the administration of TEPP they disappeared while the red blood cell ChE was at a low level of activity, being frequently less than 15 per cent of the original activity. After several weeks of daily administration of TEPP in the treatment of myasthenia gravis, the plasma and red blood cell cholinesterases were depressed to, or near, zero activity, so that the activity of these enzymes could not be used as a guide in the regulation of the dosage schedule.

The administration of atropine by any route had a moderate to marked inhibitory effect on the muscarinic symptoms due to TEPP, a slightly inhibitory effect on the central nervous system symptoms, and no effect on the nicotinic manifestations. If the muscarinic efects were moderately severe, 2 to 3 mg. of atropine intramuscularly were required every hour as long as symptoms persisted in order to provide relief. It was quite evident that in this situation the tolerance for atropine was increased to many times the dose of this drug ordinarily producing signs of over-atropinization. The central nervous system symptoms due to TEPP could be further reduced by the administration of phenobarbital.

The Effect of TEPP on the Gastro-Intestinal Tract

The gastro-intestinal symptoms that followed the administration of TEPP to both normal and myasthenic subjects were less marked and more transient than was the case with DFP. The motility of the small and large intestine, studied by intubation (3), was not as strikingly affected by TEPP as by DFP. There was only a very slight effect from a single, relatively large dose of TEPP (3 mg. in oil intramuscularly). A moderate effect upon gastro-intestinal motility appeared after the second and third doses when these were given daily, but persisted for only one-half to two hours, in contrast to three to five hours after DFP (3). The prior administration of TEPP sensitized the intestinal tract to the stimulating effect of neostigmine and pitressin, but this sensitization did not persist for as long a period of time as had been the case with DFP. The antagonistic effect of atropine on the smooth muscle contractions due to TEPP was the same as on those due to DFP.

DFP administration results in the development of rhymical con-

tractions of the intestines lasting for long periods of time, and the sensitizing effect which it has upon subthreshold doses of neostigmine and pitressin persists for one to two weeks (3). Thus, it has been a more satisfactory drug for the treatment of paralytic ileus than TEPP.

The Effect of TEPP on Neuromuscular Function in Normal Subjects and in Myasthenia Gravis

The effects of TEPP on neuromuscular function were in general similar to those of DFP and neostigmine, though resembling neostigmine more closely. The duration of the effects of TEPP was shorter than those of DFP and longer than those of neostigmine.

The intramuscular administration of 0.5 to 5 mg. of TEPP to normal subjects invariably resulted in the immediate development of fasciculations in the injected muscle. These were extremely numerous and lasted for from one to twenty minutes, being more numerous and less prolonged when the TEPP was in aqueous solution than in peanut oil. When TEPP was administered daily the duration of the local fasciculations following each injection decreased progressively from about ten minutes on the first day to one minute on the sixth. When the doses were high, generalized fasciculations soon appeared in the normal subjects, and there was often a mild generalized weakness, with increased fatigability after activity.

TEPP was injected into the brachial artery of eleven normal subjects, in amounts from 0.25 to 2.5 mg. in aqueous solution. The administration of 0.5 mg. or more of this drug by the intra-arterial route resulted immediately in the appearance of myriads of fasciculations, and moderate to marked weakness, in the injected forearm and hand. The degree and duration of the fasciculations and weakness were proportional to the dose administered. The fasciculations were maximal immediately after the injection and gradually disappeared over a period of one to two hours. A similar time course was recorded for the muscular weakness, although the return to completely normal strength was somewhat more delayed (Fig. 6). Sweating usually appeared over the injected forearm and hand despite the previous administration of 0.6 to 1.2 mg. of atropine intramuscularly. The duration of changes in function which followed the intra-arterial administration of TEPP was more than twice that of the similar effects following an

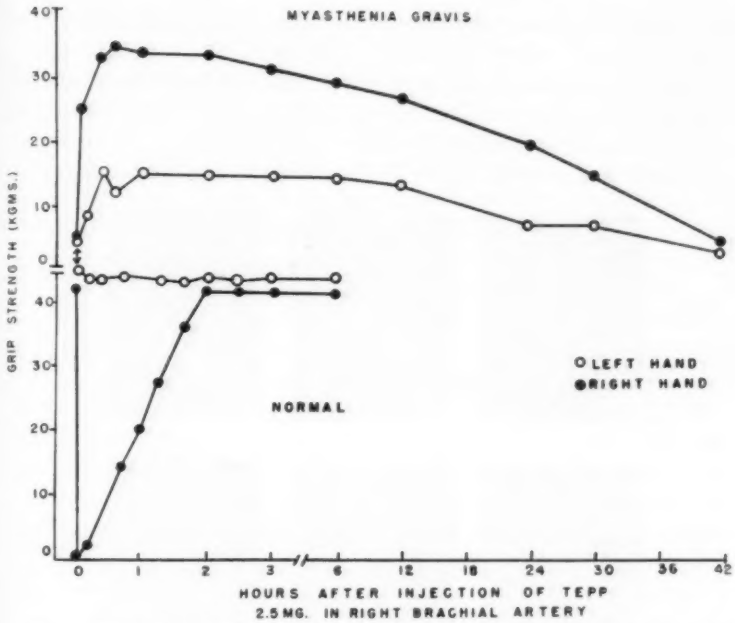


FIG. 6. The effect of TEPP administered intra-arterially on muscle strength in a normal subject (C. D.) and in a patient with myasthenia gravis (G. R.).

TABLE 6

Duration of the Effect of Anticholinesterase Agents on Muscular Strength after Intra-arterial Administration

(Hours)

DOSE	NORMAL SUBJECTS		MYASTHENIC SUBJECTS
	Fasciculations	Weakness	Increased strength
Neostigmine....(0.5–3.0 mg.)	0.1–0.5	0.3–0.7	3–5
TEPP.....(1.0–2.5 mg.)	0.5–1.0	1–2	24–48
DFP.....(0.5–2.0 mg.)	0.5–216	24–1920	24–216

equal dose of neostigmine, but much less than the duration of such changes after the injection of DFP (Table 6).

Electromyographic studies showed that the effects of TEPP, DFP,

and neostigmine on neuromuscular function in normal subjects are similar in most respects, the most striking difference being that of the duration of the observed alterations. Following the intra-arterial injection of TEPP there was no change in the voltage or duration of the muscle action potential produced by a single maximal motor nerve stimulus, but the normal diphasic potential was converted into a repetitive response consisting of a series of spikes diminishing progressively in voltage and duration. When two maximal motor nerve stimuli were applied at varying intervals of time there was no repetitive response after the second stimulus unless the interval between the two stimuli exceeded 150 milliseconds. There was a very significant reduction in the voltage of the response to the second stimulus, the reduction being greatest at the shortest interval between the two stimuli (Fig. 7). The muscle action potential responses to a train of

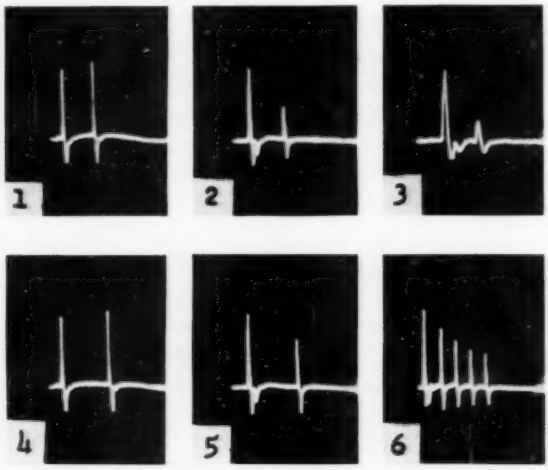


FIG. 7. The effect of TEPP on the electromyogram of a normal subject (C. D.). *1 and 4*: Responses to 2 nerve stimuli, at intervals of 45 and 60 msec., before TEPP. *2 and 5*: Responses to similar stimuli 15 min. after the injection of 2 mg. TEPP into the brachial artery, showing the depressed voltage of the second response, which is more marked when the interval between the stimuli is short. *3*: Faster sweep speed showing the repetitive character of the initial response after TEPP. *6*: Response to a train of 5 stimuli after TEPP, showing the progressive decline in voltage of successive potentials. Initial response in *1* equals 8 mV.

motor nerve stimuli at rates varying from 12 to 50 per second also showed a reduction in the response to successive stimuli. These potentials declined progressively in the same fashion as those observed after the administration of neostigmine.

The intra-arterial injection of d-tubocurarine chloride a few minutes after the administration of TEPP resulted in a prompt disappearance of the fasciculations and a more rapid rate of return of muscular strength than had occurred previously following the injection of TEPP alone into the opposite brachial artery.

TEPP was injected into the brachial artery of three patients with myasthenia gravis. In all of these patients there was moderate to severe weakness of the muscles of the upper extremities in the basal state. At the time of the injection no neostigmine had been administered for at least 6 hours. The injection of 2.5 mg. of TEPP resulted in a prompt increase in the strength of the injected forearm and hand, reaching a maximum within 10 minutes (Fig. 6). There was slight sweating below the level of the injection despite the prior intramuscular injection of 0.6 mg. of atropine. No fasciculations were observed in the injected arm or elsewhere. After the release of the pressure cuff which had been applied above the site of injection, there was a slight, but definite increase in strength of the opposite arm and also in the affected muscles generally (Fig. 6). The local increase in strength of the injected arm remained at a maximal level for a period of 6 hours and then declined slowly, reaching in about 40 hours the basal level of weakness which existed before injection. The slight increase in strength of the muscles elsewhere declined progressively over a period of 20 hours.

Electromyographic studies were carried out in all three myasthenic patients. Before the injection of TEPP the muscle action potentials in response to stimulation of the ulnar nerve revealed in each instance the characteristic defect in neuromuscular conduction which has been described in this disease (20). There was a progressive decline in the amplitude of the muscle action potentials elicited by a train of nerve impulses applied at a frequency of from ten to fifty per second. Following the intra-arterial injection of TEPP this defect in neuromuscular conduction was repaired and the action potentials in reponse to trains of nerve impulses, of the frequency mentioned, all had the same voltage (Fig. 8). In one patient with severe myasthenia gravis there

was a partial block in neuromuscular conduction before the administration of TEPP, with a reduced voltage of the action potential in response to a single nerve volley. After the injection of TEPP the voltage of the single response was increased by 25 per cent. In all instances records taken 48 hours after the intra-arterial injection of

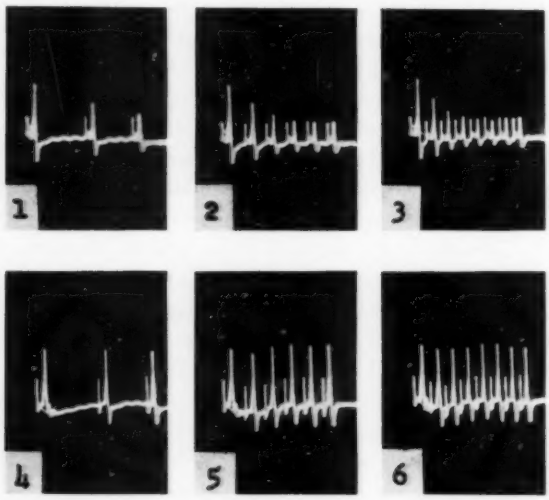


FIG. 8. The effect of TEPP on the electromyogram of a patient with myasthenia gravis (G. R.). *1, 2, and 3*: Responses to trains of 3, 6, and 8 stimuli (30, 60, and 80 per sec.) before TEPP, showing the characteristic progressive decrease in voltage of successive potentials. *4, 5, and 6*: Responses to similar stimuli 15 min. after the injection of 2.5 mg. TEPP into the brachial artery. Initial response in *1* equals 6 mV.

TEPP showed that the electromyograms had returned almost completely to the pre-injection pattern.

The effect of TEPP on neuromuscular function of myasthenic subjects following intra-arterial administration was intermediate between that of DFP and neostigmine with respect to both duration of action and degree of localization, the effect of DFP being most prolonged (Table 6) and most localized to the injected extremity.

Blocking of the Action of TEPP by the Prior Administration of Neostigmine

The administration of neostigmine shortly before TEPP blocked the effect of the latter drug upon the ChE activity of the plasma and red blood cells, as well as on the increase in muscular strength that occurred in myasthenic subjects (Table 7 and Fig. 9). The similar effect of neostigmine upon the action of DFP has been demonstrated both in regard to ChE activity (18) and nicotinic effects (5). It seems probable

TABLE 7

The Blocking Effect of Neostigmine on the Inhibition of Plasma and Red Blood Cell ChE Activity by TEPP

SUBJECT	NEOSTIGMINE INJECTED I.M. 1 HOUR BEFORE TEPP	ChE (IN PER CENT OF ORIGINAL ACTIVITY) AT TIME OF ADMINISTRATION OF TEPP (2.5 MG. I.A.)		DECREASE IN ChE 1 HOUR AFTER TEPP (IN PER CENT OF ChE ACTIVITY AT TIME OF ADMINISTRATION OF TEPP)			
				Observed		Expected*	
		Plasma	RBC	Plasma	RBC	Plasma	RBC
C. D.	None	100	100	99	60	98	62
L. K.	None	100	100	98	59	98	62
J. H.	2 mg.	45	85	27	15	93	60
L. K†	2 mg.	34	58	41	28	94	55

* From Fig. 4 (left), which records the effect on Plasma and RBC ChE activity of TEPP administered at various levels of ChE activity.

† This subject had received a previous injection of TEPP four days before. At time of neostigmine injection ChE was 43% (plasma) and 63% (rbc) of original activity. One hour after TEPP ChE was 20% (plasma) and 42% (rbc) of original activity, and four hours later 16% (plasma) and 45% (rbc) of original activity.

that the combination of neostigmine with ChE renders the enzyme insusceptible to the inhibitory action of these phosphoric acid derivatives. By the time the ChE has been released from its combination with neostigmine, the free TEPP or DFP has apparently been rendered inactive, probably as a result of hydrolysis, which may be accelerated by enzymatic activity. Mazur has demonstrated a fluorophosphatase in plasma, red blood cells, and tissues which accelerates the hydrolysis of DFP (21). A pyrophosphatase has been described in red blood cells, but it is said to require the presence of added magnesium for its complete activity (22).

This blocking effect of neostigmine was important during the treatment of myasthenic patients with TEPP. Whenever supplementary doses of neostigmine were required, it was necessary to administer the TEPP at a time when the patient was not under the influence of neostigmine in order to obtain full benefit of its effect. Therefo

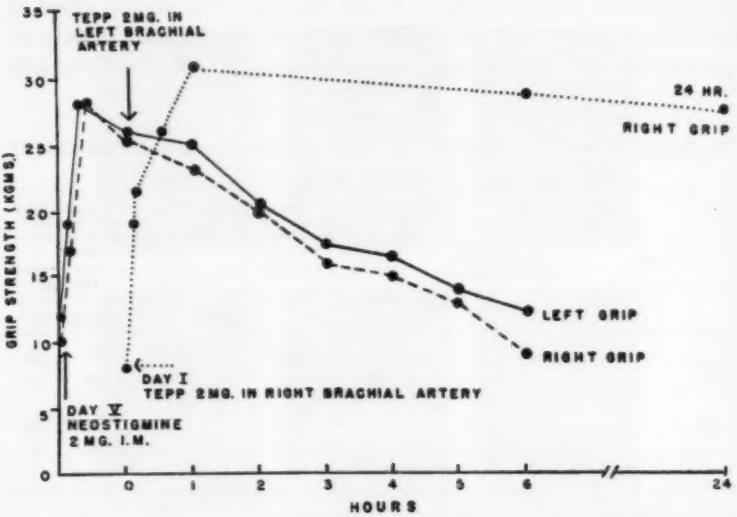


FIG. 9. The blocking effect of neostigmine on the response of myasthenic muscle to TEPP injected intra-arterially. TEPP was administered intra-arterially on Day I and caused a sustained increase in the strength of the injected extremity. On Day V, when TEPP was injected intra-arterially into the opposite extremity while the patient was under the influence of previously administered neostigmine, there was no such prolonged increase in strength.

whenever possible, TEPP was not administered until four or more hours after the last dose of neostigmine.

The Use of TEPP in the Treatment of Patients with Myasthenia Gravis

The instramuscular administration of 2.5 mg. of TEPP (in aqueous solution or in peanut oil), or the oral administration of 10 mg. of TEPP in propylene glycol, to myasthenic subjects in a basal state who had received none of the drug previously and no neostigmine for at least

six hours resulted in no increase in strength. It did cause a reduction in the ChE of the plasma and of the red blood cells to 5 and 34 per cent of original activity respectively. Repetition of the above doses of TEPP 6 to 24 hours later caused only a slight increase in general strength, and further reduction of the ChE to 3 and 25 per cent of original activity. A third dose of the drug, however, was usually followed within half an hour by a marked increase in general strength to or near the maximum obtainable after full doses of neostigmine. This increase in strength, if near maximum, was frequently accompanied by transient muscular fasciculations lasting for from several minutes to an hour. These were often most prominent in the extra-ocular muscles, resulting at times in uncontrolled jerking movements of the eyes, and in the periorbital muscles, causing tremor of the eyelids. In addition to these nicotinic effects of TEPP the patients frequently experienced symptoms due to the muscarinic and central nervous system effects despite the prior administration of 1.2 mg. of atropine orally or intramuscularly with each dose of TEPP. These symptoms included anorexia and nausea, occasional vomiting, sweating, a feeling of warmth, giddiness, uneasiness, restlessness, tremulousness, and sometimes headache.

The ChE activity of the plasma and red blood cells when the muscle power approached maximal was approximately 2 and 20 per cent of original activity respectively. This was usually the level of ChE activity when muscarinic and central nervous system effects also first appeared.

In general, the maximal effects of TEPP on muscular strength were similar to those produced by an optimal dosage schedule of neostigmine in the same patient. Occasionally, muscles which responded only partially to such doses of neostigmine showed a slightly greater degree of improvement after the largest tolerated doses of TEPP. The most striking difference between the two drugs was in the duration of the increased muscular strength, which remained maximal for 6 to 12 hours after the administration of TEPP, and then gradually declined over a period of 48 to 72 hours (Fig. 10), in contrast to maximal strength for 1 or 2 hours after neostigmine, declining over 3 or 4 hours. The doses of TEPP required to maintain maximum strength once this was attained were considerably less than the initial doses required to reach the full degree of improvement.

Twelve subjects with moderately severe and very severe myasthenia gravis, who had previously been maintained on neostigmine, have been treated with TEPP over periods up to eight months (Tables 8 and 9). The most convenient method of beginning treatment was to administer the drug orally every hour in doses of about 5 mg., with atropine, until the patient reached maximum strength. The average amount of TEPP required was 41 mg. administered orally over a period of five to twenty-

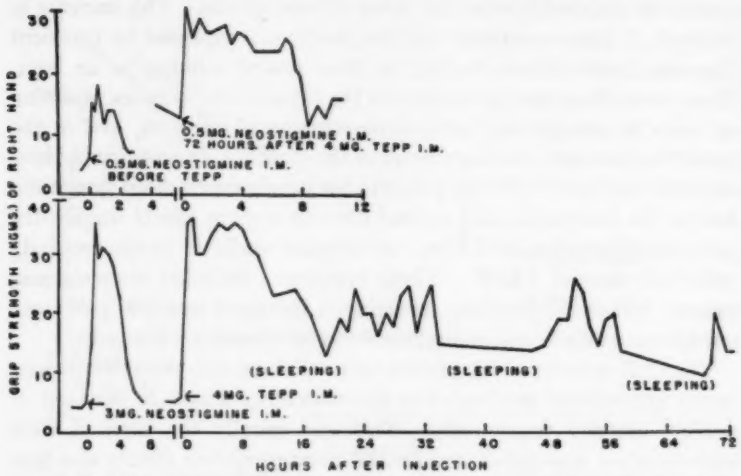

GRIP STRENGTH (KGMS.) OF RIGHT HAND
0.5 MG. NEOSTIGMINE I.M. BEFORE TEPP
0.5 MG. NEOSTIGMINE I.M. 72 HOURS AFTER 4 MG. TEPP I.M.
3 MG. NEOSTIGMINE I.M.
4 MG. TEPP I.M.
(SLEEPING)
(SLEEPING)
(SLEEPING)
HOURS AFTER INJECTION

FIG. 10. *Below*: Comparison between the effect on muscle strength of neostigmine and TEPP administered intramuscularly to a patient with myasthenia gravis (G. R.). The patient had received 2 mg. TEPP i.m. the day before the injection of 4 mg. TEPP, with no appreciable response.

Above: Potentiation of the effect of neostigmine on muscle strength by the previous administration of TEPP.

nine hours, or 7.2 mg. administered intramuscularly over two to forty-eight hours. The average daily requirement of TEPP for maintenance of strength was 16 mg. orally, or 4.8 mg. intramuscularly, divided into two or three doses, the morning dose being the largest. The required initial and maintenance doses of TEPP were in general proportional to the severity of the myasthenia gravis and to the neostigmine requirement before the administration of TEPP. Atropine was administered in doses up to 10 mg. orally per day in order to reduce the muscarinic and central nervous system effects of the TEPP to a minimum.

The differences between the dosage level of TEPP which produced maximum strength with a minimum of side effects, the dosage level which had little effect on motor power, and the level which caused prohibitive side-effects, were very narrow. Reduction in the daily maintenance dose of the drug by 3 to 4 mg. (orally) was followed by a reduction in general strength to less than half of the maximum, while increasing the daily dose by 2 to 3 mg. resulted in the appearance of troublesome muscarinic and central nervous system symptoms which could not be controlled fully by large doses of atropine (up to 10 mg. orally and 8 mg. intramuscularly over 24 hours). TEPP administration at this increased level also produced uncomfortable muscular fasciculations. In one patient an excessive dose of the drug administered intramuscularly resulted in transient symptoms suggestive of laryngeal spasm, possibly due to the occurrence of muscular fasciculations in this region. In two patients (H. L. and A. R.) the continued administration at home of excessive doses of TEPP in spite of obvious symptoms of overdose resulted in muscular weakness, as well as fasciculations, beginning in the muscles of the face, tongue, jaws, and pharynx and becoming generalized. In these patients the administration of TEPP was promptly stopped, following which the fasciculations ceased and the muscular strength gradually increased over a period of 48 to 72 hours to the maximal level that had existed previously.

The occurrence of muscular weakness and fasciculations in these two myasthenic patients following the continued administration of excessive amounts of TEPP resembles the effects of anticholinesterase agents in normal subjects. Similar effects were not observed in the other myasthenic subjects, even though most of them received excessive amounts of TEPP at some time during their regulation.

The narrowness of the optimal range of TEPP dosage necessitated careful preliminary adjustment of the treatment schedule and observation of the effect of the maintenance dosage for at least two weeks prior to discharge of the patients from the hospital. The occurrence of cumulative effects during the first week of administration of the maintenance dose frequently necessitated reduction of the dose during this time. A further reduction in the maintenance dose was sometimes possible after several weeks of daily administration of the drug. It proved of great importance that patients and their families be carefully instructed in the signs and symptoms of overdosage of TEPP, and in

TABLE 8

Description of Cases of Myasthenia Gravis Treated with TEPP

PT.	AGE	SEX	COLOR	WT.	SEVERITY OF WEAKNESS		DURATION	THYMECTOMY	HISTORY OF REMISSION	NEOSTIGMINE REQUIREMENT (mg.q.d.)		ATROPINE REQUIREMENT mg. q.d.(o)	DURATION OF NEOSTIGMINE RX.	DESCRIPTION OF BASAL STATE*
					Oculo-bulbar	Per-ipheral				(o)	(hypo.)			
				kg.			*yrs.*						*yrs.*	
L. K.	30	F	C	38	++	++	13	7 yrs. ago	Partial after thymectomy	75	0	0	12	Facial muscles weak; up and about, but with easy fatigue of arms and legs.
M. W.	41	F	W	57	++	++	10	7 yrs. ago	Partial after thymectomy	90	0	0	9	Facial and masticatory muscles weak, unable to sit up, could barely raise arms.
H. L.	52	F	W	61	+++	+++	2	1½ yr. ago	0	90	0	0.6	2	Unable to swallow semi-solids, chew, smile, speak intelligibly, raise head, or sit up.
R. N.	30	F	W	48	++++	++	8	0	0	120	0.5	0.6	8	Unable to swallow liquids; voice unintelligible; facial muscles, arms and shoulders very weak.
D. L.	19	F	W	48	+++	++++	3	0	0	120	0	0	3	Could barely swallow liquids and raise arms; unable to smile or sit up.

G. R.	30	F	W	53	+++	++++	8	0	0	180	0	1.2	3	Could swallow liquids only; unable to raise arms or legs, sit up, or roll over in bed.
G. K.	60	M	W	75	+	++++	4	0	0	135	0	1.2	2	Could barely swallow liquids, chew, smile, or get out of bed; speech unintellibible.
F. W.	29	F	W	48	++++	++++	4	1 yr. ago	0	150	0.5	1.2	3	Unable to swallow liquids, speak intelligibly, smile, raise arms, or sit up.
A. M.	33	M	C	66	+	++++	4	3 yrs. ago	0	180	0	0	3	Unable to raise head, arms, or sit up.
A. R.	45	F	W	53	+++	++++	14	0	0	225	0	1.6	10	Unable to swallow semi-solids, chew, smile, speak intelligibly, raise head, or sit up.
S. D.	38	F	W	68	+++	+++	5	0	0	300	0	0	5	Unable to swallow semi-solids, chew, smile, raise arms, or sit up.
J. H.	30	M	W	61	++++	++++	3	1 yr. ago	0	540	1.0	9.6 (h)	3	Unable to swallow liquids, speak intelligibly, smile, raise arms, or sit up. Poor response to neo-stigmine.

* All patients also had ptosis and limitation of extra-ocular movements, with diplopia, in the basal state.

TABLE 9

The TEPP Requirement of 12 Myasthenic Patients, and the Effect of TEPP on Grip Strength, on Atropine Requirement, and on Muscles Which did not Respond Fully to Neostigmine

TEPP was administered orally in propylene glycol in 2 or 3 divided doses daily, or (to G. R. and J. H.) intramuscularly in peanut oil. Grip strength is of the right hand.

	BEFORE TEPP		DURING TEPP							
	Grip Strength (Kg.)				TEPP Requirement (mg. (o) q.d.)				ATROPINE REQUIREMENT	MUSCLES THAT DID NOT REACH FULL STRENGTH AFTER NEOSTIGMINE OR TEPP
						Maintenance				
	Basal	Max. after Neostig.	Basal	Max.	Initial*	Half Max. Strength	Max. Strength	Max. Strength and Marked Side Effects		
					hrs.				*mg.(o)q.d.*	
L. K.	13	31	27	32	40 (29)	8	12	15	0	EOM
M. W.	2	25	24	27	22 (5)	13	16	18	6	EOM
H. L.	6	25	23	28	30 (6)	13	16	18‡	8	EOM
R. N.	7	26	21	29	26 (8)	8	11	13	7	EOM, facial, speech, extensors of forearms, hands, fingers
D. L.	6	25	24	33	46 (28)	11	14	16	6	EOM, extremities
G. R.	3	35	30	39	46 (13) or i.m. 7.2 (48)	12	18 or i.m. 4.2	20	6	EOM
G. K.	16	29	31	33	36 (24)	13	16	19	6	EOM, speech, legs
F. W.	1	25	18	27	60 (72)	13	16	18	7	EOM, extremities
A. M.	7	45	40	50	50 (12)	17	20	22	10	None
A. R.	1	15	15	26	50 (12)	14	17	19‡	11	EOM, extremities
S. D.	9	26	23	30	45 (8)	12	14	16	12	EOM, facial
J. H.	6	19	17	20	7 i.m. (2)	12†	18†	21†	9.6(h)	All muscles†
Average	6	27	24	31	41 (20)	12	16	18	7	

* Amount of TEPP required to increase the basal strength when administered over the indicated time.

† Patient J. H., a very severe myasthenic who responded poorly to large doses of neostigmine, was the only one who could not be regulated on TEPP alone, requiring, in addition, 240 mg.(o) of neostigmine daily to maintain respiration.

‡ These doses of TEPP administered to patients H. L. and A. R. resulted in generalized muscular weakness, in addition to marked side effects. TEPP was discontinued in these two patients and neostigmine resumed.

the importance of adjusting the dose in accordance with the response of the patient.

When the drug was discontinued the patients' strength declined gradually to the original basal state in about 48 hours. If the drug was resumed at this point considerably more than the maintenance dose had to be given to regain maximal strength, although not as much as was originally required. There was no change in the severity of the myasthenia gravis or in its response to neostigmine after cessation of treatment with TEPP.

When TEPP was administered in amounts sufficient to increase the muscular strength of patients with myasthenia gravis above the basal level but not to a maximum there was usually a significant potentiation of the effect of subsequent doses of neostigmine administered either intramuscularly or orally. The degree of improvement in muscular power after small doses of neostigmine was in most cases significantly increased and the effect lasted two or three times longer than before the administration of TEPP (Fig. 10). This potentiation of neostigmine action could be demonstrated for only about forty-eight hours after TEPP administration was discontinued, at which time there was still a pronounced reduction in the ChE activity of both red cells and plasma. On the other hand, when sufficient TEPP had been administered to bring about a maximum increase in muscular strength, the administration of neostigmine resulted in no further improvement, even in muscles which had not responded fully to the administration of TEPP. In three patients with severe myasthenia gravis (R. N., F. W., and A. R.) the administration of 1 to 2 mg. of neostigmine intramuscularly at a time when the patients were at maximum strength following TEPP produced a generalized weakness which was very striking. There was difficulty in swallowing, impairment of speech, sagging of the jaw, marked ptosis, and severe weakness in the extremities. In two patients there was a gradual return of strength over a period of four hours after the administration of neostigmine.

In the third patient (A. R.) failure to recognize the weakness as being due to excess of anticholinesterase drug nearly led to a fatal outcome. This patient was regulated on 18.5 mg. of TEPP (orally) daily, and 10 mg. of atropine. On this schedule her general strength and endurance were better than when she had been taking neostigmine, and she had no

[Patient J. R., a very severe myasthenic who responded poorly to large doses of neostigmine, was the only one who could not be regulated on TEPP alone, requiring, in addition, 240 mg.(o) of neostigmine daily to maintain respiration.

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symptoms suggestive of overdose of TEPP during the week that she was in the hospital. Two days after discharge, however, she developed nausea, sweating, uneasiness, and weakness of the muscles of the face, tongue, and pharynx, accompanied by fasciculations. The patient took an additional 2 mg. of TEPP in the belief that this would increase her strength, and she became still weaker. Although she had been advised against taking neostigmine while on her full TEPP schedule she then took 30 mg. of neostigmine by mouth. This was followed by generalized weakness and muscular fasciculations, with inability to swallow or speak. She was rushed to the emergency room where the physician on call administered 1 mg. of neostigmine intramuscularly. This was followed by sweating, salivation, generalized muscular fasciculations, profound generalized weakness, and finally cessation of respiration with loss of consciousness. An endotracheal catheter was inserted, and artificial respiration and oxygen administered. The patient was placed in a Drinker respirator, and soon regained consciousness. She was given no medication during the next 24 hours, except atropine intramuscularly. Her strength gradually increased to the maximal level that had existed previously, and the muscular fasciculations disappeared.

Of the twelve myasthenic patients who have been treated with TEPP, neostigmine has been withdrawn during the time of administration of TEPP in all except one patient (J. H.), who had critically severe myasthenia gravis and required enormous doses of neostigmine. TEPP had to be discontinued in two patients (H. L. and A. R.) who developed muscular weakness following the administration of excessive amounts of this drug, and neostigmine was resumed. The remaining nine patients were maintained on TEPP for periods of one to eight months, and all had better general strength and endurance than during the administration of neostigmine. Three of the nine patients (R. N., D. L., and S. D.) had moderately severe nausea due to TEPP which was not entirely prevented by atropine. After being maintained on TEPP for periods of one to two months they requested that they be returned to neostigmine, in spite of the increased strength which the TEPP had afforded. The remaining six patients have been satisfactorily maintained on TEPP for four to eight months and prefer this drug to neostigmine. They are continuing to receive TEPP at the present time.

Effects of TEPP Other Than Those Attributable to its Anti-Cholinesterase Activity

The twelve patients with myasthenia gravis who received TEPP daily for periods up to eight months were observed carefully and no effects of the drug were noted other than those attributable to its anticholinesterase activity. There was no change in temperature or body weight. No alterations were noted in the hematocrit, hemoglobin, red cell count, white blood count or differential formula. Determinations were made at intervals of blood non-protein nitrogen, fasting blood sugar, chloride, carbon dioxide combining power, calcium, phosphorus, alkaline phosphatase, serum proteins, and bilirubin, but no changes were detected. The following tests of hepatic and renal function remained normal: thymol turbidity, cephalin flocculation, bromsulfalein retention, phenolsulphonphthalein excretion and urinalysis. The basal metabolic rate remained unchanged and teleoroentgenograms were normal. In one patient (G. R.) there was a slight transient prolongation of the PR and QRS intervals of the electrocardiogram.

DISCUSSION

Dubois and Mangun (14) were the first to point out that the insecticide, hexaethyl tetraphosphate (HETP), which had been known to have strong nicotinic effects, has a potent inhibitory effect on mammalian as well as insect ChE both in vitro and in vivo. Westerberg and Luros (22a) found that the administration of HETP to patients with myasthenia gravis resulted in an increase in strength. Mangun and Cleveland (17) demonstrated that HETP is really a mixture containing neutral esters which account for the major portion of its biologic activity, and that one active neutral ester is TEPP. Of the compounds of this group of phosphoric acid derivatives which appear to derive their activity from the unstable pyrophosphate type of linkage, TEPP seems to be the best tool for the continuation of studies on various anticholinesterase agents in the human subject.

Brauer (23) described experiments which led him to conclude that a stable compound was formed between ChE and a phosphorus-containing moiety of HETP or TEPP. While these studies in vitro were said to demonstrate irreversibility of the inhibition of plasma ChE under the conditions present (23), other experiments in vivo showed that the

brain ChE activity of rats recovered several times more rapidly after the administration of TEPP than after comparable doses of DFP (24). The experiments described in the present communication demonstrate a type of recovery curve of red cell ChE in vivo compatible with partial dissociation of the TEPP-ChE complex during the early period following administration of the inhibitor. After twenty-four to forty-eight hours the recovery curve is slow and at a rate compatible with the formation of new red blood cells, suggesting that at this time the return of ChE activity is by the formation of new enzyme and not by reactivation of inactivated enzyme. These observations pointing to a limited dissociation of the TEPP-ChE complex in vivo during the early period after formation of the complex have been supported by experiments in vitro, and serve to explain the shorter duration of the clinical symptoms produced by TEPP as compared to DFP. Since the red cell ChE is still greatly depressed when symptoms due to TEPP disappear, it is probable that the tissue ChE activity returns more rapidly after depression by TEPP than does that of the red blood cells, and that a speedy partial return in the first few hours might raise the level of tissue ChE to a range compatible with normal function, even though return to completely normal activity might be delayed for several days.

This partial dissociation of the inhibitor-enzyme complex would also afford an explanation of the less striking potentiation of repeated doses of TEPP in human subjects as compared to DFP. With TEPP the sensitizing effect of a given dose declines rapidly over a few days, in contrast to a period of weeks following DFP. This has enabled the daily administration of TEPP in larger doses than is possible with DFP.

The results of TEPP administration support the hypothesis previously set forth (5) that the distribution of the physiological activity of these ChE inhibitors depends to a certain degree on their physical properties. The lipoid-water partitions of DFP, TEPP, and neostigmine, as demonstrated in the experiment employing peanut oil, suggested that the effects of TEPP would be likely to resemble qualitatively those of neostigmine more closely than those of DFP. Such has been the case in the studies on human subjects, and the observations make it probable that this anti-ChE agent will be useful in the treatment of myasthenia gravis.

It may be pointed out that TEPP, like DFP, has no direct excitatory

effect on muscle, in contrast to the direct effect of neostigmine described by Riker and Wescoe (25). The ability of TEPP to restore power to myasthenic muscle bears a general resemblance to the effects of neostigmine. This might suggest that, since TEPP exerts its effects only by virtue of its anticholinesterase activity, the effect of neostigmine on myasthenic muscle may likewise be due primarily to its anticholinesterase activity, rather than to its ability, as a weak choline ester, to stimulate muscle directly. However, certain differences between the action of TEPP and neostigmine must be noted. A large initial dose of TEPP is required before its physiological effects are produced, and the range of effective maintenance dose between that which produces no observable effect and that which causes distressing symptoms is narrow. In animals there is a very narrow range between the maximum dose of TEPP which is safely tolerated, and the dose which is invariably fatal. These characteristics of TEPP action are in contrast to those of neostigmine. TEPP would appear to exert its effects by depressing the ChE activity of the tissues below a threshold at which changes in function begin, and by maintaining this degree of depression. The difference between this threshold and the level of ChE activity below which serious and even fatal functional alterations occur, appears to be narrow. The mode of action of neostigmine, if similar, would seem to entail a qualitative difference in its effect on ChE enzymes and in the production of physiological effects.

The electromyographic observations in the normal subjects provide certain points of interest. Qualitatively they are similar to those seen after neostigmine and DFP. When two maximal nerve stimuli are applied, the muscle action potential in response to the second stimulus is greatly diminished in the presence of these ChE inhibitors. Full recovery does not take place until the stimuli are well over 250 msec. apart. As the initial response is not changed in voltage this phenomenon of depression is a result of the changes associated with the passage of a single nerve impulse at the neuromuscular junction. Its time course is compatible with the accumulation and gradual dissipation of acetylcholine and favors the theory of chemical mediation at the neuromuscular junction.

Eleven patients with myasthenia gravis have now been treated with TEPP alone for periods up to eight months, and one patient has been

treated with TEPP supplemented by neostigmine. The initial regulation of TEPP dosage in the treatment of myasthenia gravis is rather difficult, due to the narrow range between the amount which produces a maximum effect on muscular function and that which causes unpleasant and potentially dangerous muscarinic, nicotinic, and central nervous system effects. The daily maintenance dose must be determined with care, for if it is much below the threshold amount for good, steady muscular power throughout the twenty-four hour period the strength drops to a minimal level. It is helpful to know that under these circumstances, if the strength is above the basal level, the response to neostigmine will usually be enhanced and prolonged. It has been found possible, following the initial regulation in the hospital, to administer maintenance doses of TEPP to patients on an out-patient basis, and six of twelve patients who have received the drug have preferred it to any other medication because of the maintenance of a steady increase in muscular power throughout the day and night.

SUMMARY

1. Tetraethyl pyrophosphate (TEPP) is a more potent inhibitor of human plasma, red blood cell, brain, and muscle cholinesterases in vitro than either di-isopropyl fluorophosphate (DFP) or neostigmine. TEPP has a greater affinity for plasma ChE than for the other cholinesterases, which are inhibited to a lesser, and approximately equal, degree. TEPP combines with ChE over a period of several minutes, following which ChE activity may be restored very slowly and incompletely during prolonged incubation, especially at 37°C.

2. The administration of TEPP to human subjects by any route causes a rapid fall of the ChE activity of the plasma to near zero, and a somewhat less striking depression of the ChE activity of the red blood cells, which can be reduced to near zero by repeated doses of TEPP or by a single very large dose. Approximately four times as great an oral dose is required to produce the same effect as when the drug is administered intramuscularly or intravascularly.

3. Within one to two hours after the administration of TEPP the ChE activity of the plasma and red blood cells begins to return toward normal, the former at a rate of 19 per cent of original activity during the first day and falling off to 4 per cent after the fifth day, the latter at a rate of 10 per cent during the first day and 2 per cent after the second

day. The rates of return of ChE activity are more rapid following depression by TEPP than by DFP during the first twenty-four hours after administration, probably due to partial dissociation of the TEPP-ChE complex during this time.

4. The muscarinic, nicotinic and central nervous system effects which follow the administration of TEPP to normal and myasthenic subjects have been described. TEPP, like neostigmine, has more marked nicotinic effects after systemic administration, and less marked central nervous system effects, than does DFP. This may be due to the greater solubility of TEPP and neostigmine in aqueous than in lipoid medium, and to the greater solubility of DFP in lipoid medium. In general, the maximal effect of TEPP on the muscular strength of myasthenic patients is similar to that produced by optimal amounts of neostigmine, except that the effect of TEPP is much more prolonged, the increased strength being fully sustained for 6 to 12 hours and gradually declining over a period of 48 to 72 hours.

5. Eleven patients with moderately severe and very severe myasthenia gravis who had previously been maintained on neostigmine have been treated with TEPP alone, and one with both TEPP and neostigmine. The average amount of TEPP required to reach maximum or near maximum strength has been 41 mg. administered orally over a period of five or more hours. The average daily dose for maintenance of strength has been 16 mg. orally in two or three divided doses. The differences between the dosage level of TEPP which produces a maximum increase in strength with a minimum of side-effects, the dosage level which has very little effect, and the amount which causes prohibitive side-effects, are very narrow. This necessitates careful preliminary adjustment of the treatment schedule, and careful observation.

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HISTOCHEMICAL STUDIES ON NUCLEIC ACID PHOSPHATASE[1]

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I. SPECIFICITY OF THE REACTION

Gomori (1) and Takamatsu (2) have developed a technique whereby the presumptive site of phosphatase activity can be demonstrated in tissue sections. This technique has been applied by many different investigators in a variety of studies. In view of the wide use of this procedure, it is surprising how little effort has so far been expended toward making this method applicable to the study of those specific phosphatases that are of prime interest to biochemists. Gomori, using fixed and paraffin embedded tissue, found that the histological pattern of presumptive enzyme activity was the same for all the phosphate substrates which he used. He concluded, therefore, that only the non-specific phosphomonoesterase survived the rigors of the histological technique. The variation in activity which he found in sections from various organs, corresponded satisfactorily to the organ distribution of phosphomonoesterase demonstrable by conventional techniques (3).

Glick (4) using adenosinetriphosphate as substrate on sections of tissue fixed in cold acetone and sectioned either frozen or after being embedded in paraffin, claimed that some of the phosphate liberating activity which occurs under these circumstances is attributable to the specific adenosinetriphosphatase (ATPase). The evidence for such specific enzyme activity, however, was merely that activity was found in some tissues in which the phosphomonoesterase is sparingly present. Moog (5) points out that the specific ATPase is 70% inhibited by exposure for one hour to cold acetone, and doubts whether appreciable activity of this enzyme could be demonstrated after 24 hours fixation.

Süllmann (6) using sections of alcohol fixed and paraffin embedded cornea finds some variation in the location of the histochemical reaction

[1] This work was supported in part by a grant in aid from the American Cancer Society through the Committee on Growth of the National Research Council.

product depending on the substrate which he used. His results suggest that some moiety of the various specific phosphatases may survive in the fixed tissues under special conditions. Such specific phosphatases could conceivably be distinguished from the non-specific phosphomonoesterase by their response to various activators and inhibitors, but no such demonstration of specificity has, so far, been described.

The one solid study in which the histochemical technique has been applied to a specific phosphatase system is that of Krugelis (7) who used ribose and desoxyribose nucleic acids as substrates. Most of the phosphate groups in these substances are bound in di-ester linkages and are hence inaccessible to the phosphomonoesterase enzyme.[2] Krugelis hoped, therefore, that activity of specific enzyme systems might be revealed in these substrates. She found that with ribose nucleic acid as substrate, the reaction product was deposited only in the cytoplasm. With desoxyribose nucleic acid (depolymerized by preliminary incubation with nuclease) the reaction product was deposited only in the nucleus, and, for cells in mitosis, only in the chromosomes. This high specificity in the location of the reaction product may be taken as strong evidence for the specificity of the enzyme systems attacking these substrates.

The present study is an attempt to extend the investigation of Krugelis by the use of frozen sections[3] of fresh unfixed tissue, and by the application of specific activators to the enzymatic reaction. No good reason has appeared in the literature as to why the Gomori technique should be applied exclusively to fixed tissues, nor why the investigators who have used this technique should confine their interest to those enzymes that survive in part the rigors of fixation. On the other hand, the use of activators or inhibitors might afford much

[2] According to Gulland (8), one quarter of the phosphate groups of yeast nucleic acid are bound by monoester linkages. Schmidt (9) reports that thymus nucleic acid in its native highly polymerized state is not attacked by the acid phosphomonoesterase obtained from prostate. After depolymerization by nuclease, he finds that one quarter of the phosphate groups are liberated by the monoesterase. The attack on this substrate was, however, quite slow even when the substrate and the enzyme were both present in high concentration.

[3] Frozen sections have an advantage over unsectioned fresh tissue in being more readily penetrated by substrate and reagents.

stronger evidence of enzyme specificity that has heretofore been obtained in studies of this type.

METHOD

Fresh frozen sections of rat's kidney were incubated at 37°C for 3 to 18 hours in substrate solution containing 0.1M barbiturate buffer pH 8.5–9.5, 0.03M $CaCl_2$, and 0.1% substrate.[4] After termination of incubation, the sections were washed in distilled water, immersed for 3 minutes in a solution of cobalt nitrate, washed again and then immersed for 5 minutes in a solution of ammonium sulphide. A positive reaction consists in the deposit of black granules in the section. After a final wash the sections were mounted on slides, dehydrated and cleared. As substrates we used yeast nucleic acid (Schwartz), desoxyribose nucleic acid (prepared from rat liver by the method of Brues, Tracey and Cohn (10)), and for comparison glycerophosphate, hexose diphosphate, adenosine triphosphate (from rabbit muscle by the method of Needham (11)).

RESULTS

I. *Relation of Nucleic Acid Phosphatases to Non-specific Alkaline Phosphatase*

In preliminary experiments the acid phosphatase technique of Gomori (12) was compared with the alkaline phosphatase technique outlined above. In the acid phosphatase technique, lead ions are used to precipitate the liberated phosphate. We found that, on fresh frozen sections, the acid phosphatase technique gave unsatisfactory results in that a heavy precipitate formed in the tissue in many instances even in the absence of substrate.

With the alkaline phosphatase technique, control sections of rat's

[4] The histochemical reaction depends upon the precipitation of calcium phosphate at the presumptive site of splitting of inorganic phosphate from the substrate. Since calcium phosphate is not completely insoluble, some diffusion of the reaction product before precipitation is almost inevitable. If a few drops of a solution of Na_3PO_4 are added to the substrate solution to the point of formation of a just perceptible cloud, and the solution is then allowed to stand at 37°C for an hour, and then is filtered at the same temperature, before addition of the frozen sections, the diffusion of the reaction product is much reduced, and the crispness of the final histological picture much enhanced.

kidney incubated without substrate were satisfactorily free of deposit, and sections incubated in the presence of glycerophosphate, hexose diphosphate, ATP, and ribose nucleic acid showed heavy deposits. Contrary to the findings of Gomori on fixed tissue, the histological pattern of the deposit in the frozen sections, unfixed before exposure to the substrate solutions, presented different patterns for each substrate presented. Desoxyribose nucleic acid presented to the tissue in its native highly viscous state failed to yield any deposit. However when the desoxyribose nucleic acid was depolymerized either by being exposed for 3 hours to boiling $\frac{N}{10}$ HCl solution or by incubation with desoxyribose nuclease[5] for 24 hours at 37°C, and subsequently to boiling $\frac{N}{10}$ HCl solution for one hour, a suitably acting substrate was obtained. Exposure to nuclease alone did not render the desoxyribose nucleic acid attackable by the tissue. Exposure of ribose nucleic acid to ribose nuclease and to boiling $\frac{N}{10}$ HCl did not alter its characteristics as substrate for the histochemical reaction. In all the experiments that follow desoxyribose nucleic acid was used in the depolymerized condition.

Comprison of the histological distribution of deposit yielded with the two nucleic acids as substrates revealed that in the rat's kidney ribose nucleic acid is attacked both in the nuclei and in the cytoplasm, while desoxyribose nucleic acid is attacked only in the nucleus. The results of these experiments which are largely in accord with those of Krugelis indicate that the tissue distinguishes between the two nucleic acids and that, therefore, the results of the experiments can be referred to highly specific phosphatase systems.

II. *Specific Activators*

Having established the validity of the technique on frozen sections of rat's kidney, we attempted to apply the same technique to the rat's cornea. Preliminary experiments revealed no deposit in the corneal tissues when incubated in solutions with nucleic acid as substrate such

[5] The nuclease preparations were generously supplied to us by Dr. Jessie Greenstein of the National Cancer Institute, Bethesda, Md.

as in previous experiments yielded satisfactory preparations with rat's kidney sections. It had been our habit in these experiments to incubate some 10–20 sections of tissue in 20 cc. of substrate solution. Since the rat's cornea is less than 0.2 mm. thick, the total volume of tissue in a cross section of cornea is very much less than in a cross section of kidney. It appeared possible, therefore, that some necessary co-factor for the enzymatic reactions might be eluted from the corneal sections. Experiments were therefore made with increasing numbers of corneal sections per cc. of substrate solution, and it was found that as the relative volume of tissue to substrate solution increased, some activity began to appear in the preparations.

As a first step toward identification of such potential co-factors, a solution was prepared containing those elements of Tyrode solution which were not incompatible with the phosphatase reaction.[6] It was found that the addition of as little as 3 drops of this solution to 20 cc. of substrate solution sufficed to activate the reaction (Fig. 3). Further experiments revealed that neither sodium chloride nor magnesium chloride were required in the activating solution. (It should be noted that the substrate solution itself contains abundant sodium and chloride ions.) Potassium chloride alone and glucose alone each yielded some activation but a more abundant deposit in the histochemical test was generally achieved when both these substances were added (Fig. 2). Maximal activation was achieved with glucose in a concentration of .001% in the substrate solution, and the activity was apparently unchanged by a further increase of glucose up to 0.1%. Maximal activation was achieved with KCl in a concentration of .0003M in the substrate solution, and the activity was apparently unchanged by a further increase of KCl up to .03 M. With reasonable assumptions regarding the concentration of glucose and potassium in the rat's kidney, one

[6]

NaCl	0.8 gm
KCl	0.02 gm
$MgCl_2$	0.01 gm
Glucose	0.1 gm
Water	100 cc

Calcium chloride was not included since this was already present in the substrate solution. Phosphate and bicarbonate were omitted as incompatible with the phosphatase reaction and because the substrate solution was already buffered with barbiturate.

can readily find that with 10–20 sections of rat's kidney, each approximately 20 mm² in area and 25 microns thick, suspended in a total substrate volume of 20 cc., the necessary activating concentrations for these two substances would be approached. The fact that no or only feeble activity was demonstrable in corneal sections without the addition of glucose and potassium is evidence that in this tissue also the

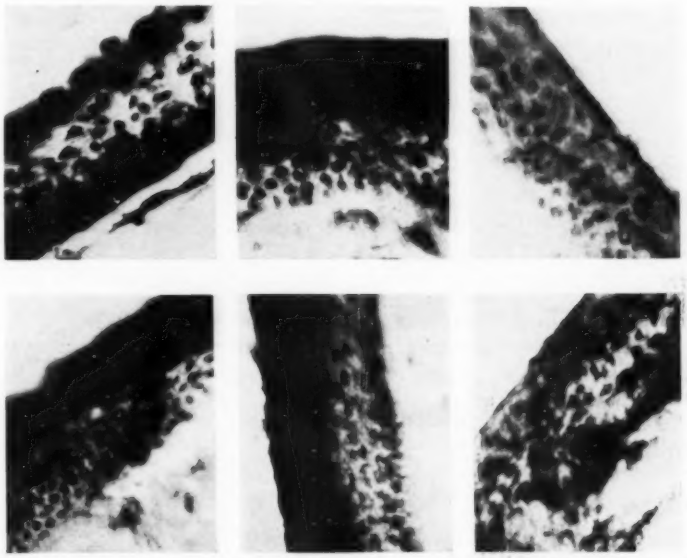

FIG. 1. Phosphatase activity in the rat's corneal epithelium with various substrates, each substrate used in concentration of 0.1%. Upper left, coenzyme I. Upper center, adenylic acid. Upper right, ATP. Lower left, hexosediphosphate. Lower center, sod. glycerophosphate. Lower right, ADP.

activity when demonstrable was not attributable to the non-specific alkaline phosphatase of Gomori. The latter enzyme does not require such activation, and fresh frozen sections of rats' cornea show an abundant activity with glycerophosphate as substrate without the addition of glucose or potassium chloride to the substrate solution (Fig. 1).

The requirements for glucose and potassium supplement to the substrate solution given above were those which we regularly found when

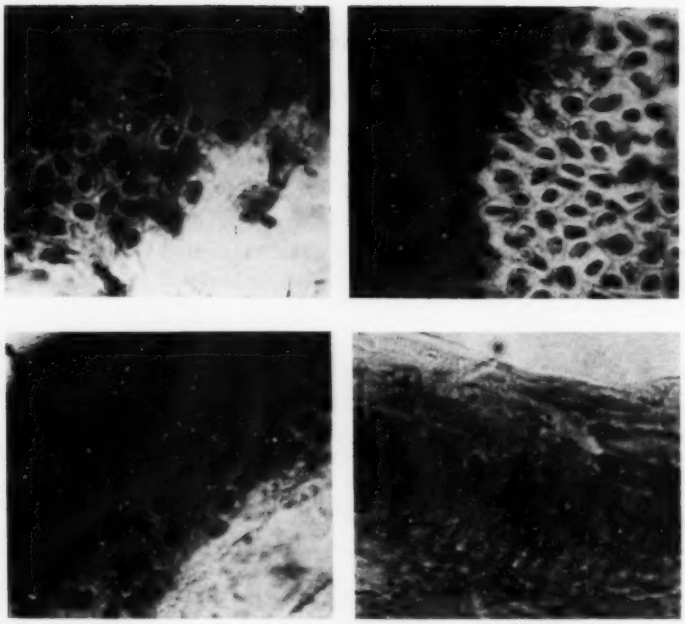

FIG. 2. Ribonuclease activity in rat corneal epithelium: pH 9.4 potassium and dextrose as activators. Upper left, ribonucleic acid 0.1% + KCl 0.0001%. Upper right, ribonucleic acid + dextrose 0.002%. Lower left, ribonucleic acid + KCl 0.0001% + dextrose 0.001%. Lower right, ribonucleic acid without KCl or dextrose.

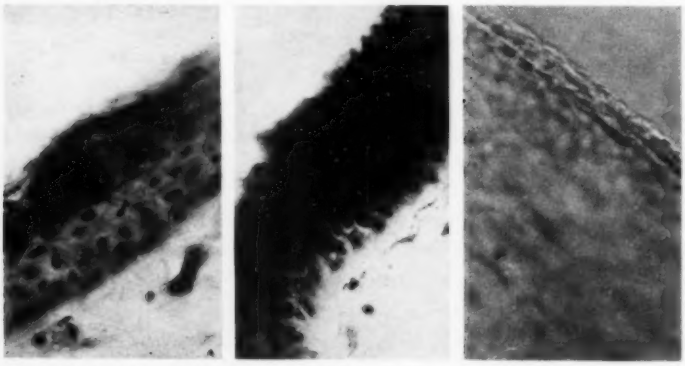

FIG. 3. Phosphatase activity at pH 9.4 in rat corneal epithelium Tyrode as activator. Left, thymonucleic acid + Tyrode. Center, ribonucleic acid + Tyrode. Right, Tyrode.

animals were used that had been in our laboratory on a regular diet of Purina dog chow (Checkers), for a period of 2 weeks or more. When animals were used immediately on delivery from the supplier, the results in testing for nucleic acid phosphatase were often erratic in that activity could be demonstrated in some batches of animals without the addition of potassium and glucose to the substrate solution. It is to be remembered in this connection that the corneal epithelium contains glycogen. The erratic results on animals whose previous diet was uncontrolled may perhaps be connected with variation in the amount or availability of endogenous glycogen stores. We have made no attempt to analyze the possibility of such a nutritional factor being content, for the purposes of the present study, with the achievement of a reproducible method of demonstrating nucleic acid phosphatase activity. Under these conditions the deposit obtained on the histochemical test, and hence the presumptive site of enzymatic activity was, for ribose nucleic acid, both in the nucleus and in the cytoplasm of the corneal epithelium, whereas that for (depolymerized) desoxyribose nucleic acid activity, in the cornea as in the kidney, was limited to the nucleus.

The requirement of glucose as an activator for the splitting of phosphate from nucleic acid appeared to us most surprising. One would not expect that metabolic energy should be required for such a process. Moreover the concentration of glucose required for maximal activation was so very low (0.001%) that it could hardly be an effective source of energy if energy were required for the reaction. Greenstein, in studies on nucleic acid phosphatase in tissue brei, found that for some tissues the enzymatic activity was enhanced by preliminary dialysis and subsequent replacement of inorganic salts. The lower rate of liberation of inorganic phosphate in the undialysed brei indicates, as Greenstein (13) has pointed out, the presence in the brei of a diffusible substance which acts either as an inhibitor of the nucleic acid phosphatase or as an acceptor of the liberated phosphate. Accordingly Greenstein has suggested[7] that the activation which we find on addition of glucose might be due to a role of this substance as a link in a chain of phosphate transfer, accepting phosphate from nucleic acid and yielding it up as inorganic phosphate in the presence of hexokinase. A close linkage

[7] Personal communication.

of the required set of transphosphorylases in the relatively undisturbed cellular structure in frozen sections might lead to an increase in the rate of liberation of inorganic phosphate in organized tissue, while in the disorganization of tissue brei, the presence of a phosphate acceptor might decrease the rate of inorganic phosphate liberation.

In order to explore these interesting possibilities it would be necessary to apply rigorously quantitative analyses to the rate of phosphate liberation under varying conditions, using techniques similar to those applied by Greenstein. Histochemical techniques are highly unsuited for quantitative analysis and can reveal only very large changes in the rate of phosphate liberation. The following experiments cannot, therefore, be regarded as conclusive but are, nevertheless, of possible interest.

To test whether the role of glucose as an activator of the phosphatase reaction was that of an energy donor or of a phosphate acceptor, we have compared the effects of adding hexose diphosphate in place of glucose to our substrate solution. In concentration of 0.1% to 0.01% hexose diphosphate acts as substrate for phosphatase yielding an appreciable deposit in the sections. With concentrations of 0.001% or lower, no visible deposit was obtained up to 24 hours of incubation. Suh concentrations, however, corresponded to those in which glucose achieved its maximum activation when added to substrate solution containing 0.1% nucleic acid. Consequently we added .001% hexose diphosphate to the standard substrate solution of nucleic acid. Some increase in the deposit was produced by the hexose diphosphate when these preparations were compared with those containing neither glucose nor hexose diphosphate. However, the maximum activation achieved with hexose diphosphate in these concentrations was considerably less than achieved by glucose in equimolar concentration. Since hexose diphosphate itself acts as substrate in higher concentration, there can be no doubt as to its ability to penetrate within the cells of the frozen section. As an energy donor hexose diphosphate would be expected to be more effective, as a phosphate acceptor less effective than glucose. The results are in accord with the supposition that the role of glucose in this reaction is that of a phosphate acceptor.

Adenosine triphosphate,[8] adenosine diphosphate,[9] and muscle ade-

[8] Prepared from rabbit muscle by the method of Needham (11).

[9] Prepared from ATP by the method of Colowick and Kalckar (14).

nylic acid (Schwartz) were each tried in concentrations of 0.001% at which level they do not themselves serve as effective substrates. No effect was obtained with any of these agents added to substrate solution containing 0.1% nucleic acid either in the presence or in the absence of glucose. These substances are, therefore, either not required for the transphosphorylation process or, alternatively, are already present in the tissue in sufficient amounts.

If glucose acts as a link in a transphosphorylating system hexokinase would probably be involved in the reaction. Colowick and Price (15) reported that hexokinase was activated by reduced cozymase, inactivated by oxidized cozymase. In a subsequent note, Colowick (16) reported that he was unable to repeat the original experiments. No explanation has been offered so far for this discrepancy. We used a preparation of cozymase obtained from Schwartz (30% cozymase, 70% unidentified constituents) and found that on addition of this material to the substrate solution at a concentration of 0.001%, the rate of liberation of phosphate was enhanced if the "cozymase" had previously been reduced by hydrosulfite, and was diminished if the "cozymase" had previously been oxidized by methylene blue according to the method of Green and Dewan (17). The addition of the oxidizing or reducing agent itself to the substrate solution in corresponding amounts produced no measurable effect on the nucleic acid phosphatase reaction.

In testing various oxidizing and reducing agents on the nucleic acid phosphatase anomalous results were obtained with quinone and with ascorbic acid. Each of these produced an apparent activation of the phosphatase, but each when added to the control solution in the absence of organic phosphate substrate yielded a deposit in the tissue. The anomalous behavior of quinone appears probably due to the binding quinone to the tissue, and the subsequent binding of cobalt ions by the quinone. The anomalous behavior of ascorbic acid to which attention has already been called by Lassek (18) appears to be due to the oxidation of ascorbic acid and the subsequent splitting off of oxalate. In the presence of calcium in the substrate solution calcium oxalate would be deposited. Subsequent treatment with cobalt nitrate and then with ammonium sulfide would give a deposit of cobalt sulfide in place of the calcium oxalate. The reaction is of interest as a possible means for the study of ascorbic acid oxidase in tissue sections.

SUMMARY AND DISCUSSION

The application of the histochemical alkaline phosphatase technique of Gomori to fresh frozen sections reveals activity for ribose nucleic acid as substrate both in the nucleus and cytoplasm. With (depolymerized) desoxyribose nucleic acid as substrate, activity was found only in the nuclei.

Fresh frozen sections of rat's cornea showed no activity with either of these substrates in the absence of glucose and potassium chloride, but strong activity in the presence of very low concentrations of these two substances. Since the non-specific phosphomonoesterase does not require these activators in order to attack glycerophosphate, it is concluded that under the conditions of these experiments, the nucleic acids are attacked solely by enzyme systems specific for these substrates.

Our findings in respect to the activating influence of very small amount of glucose are in accord with the suggestion of Greenstein that the attack of the tissue on nucleic acid may involve as its first step a transphosphorylation rather than a simple dephosphorylation. The histochemical technique does not readily lend itself to such quantitative appraisal as is necessary in order to explore the mechanism of activation and further studies by other techniques are required. Nevertheless it should be pointed out that enzymatic histochemical tests applied to tissue in the relatively undisorganized state of fresh frozen sections can, potentially, reveal the integrated action of highly complex systems of enzymes that may be decomposed into apparently unrelated elements in disorganized tissue brei. Thus the histochemical technique, a blunt tool in respect to quantitative measurements can, nevertheless, furnish evidence of enzymatic integrations that are lost in the more conventional procedure.

The possibility of enzymatic transphosphorylation from nucleic acid to glucose raises interesting questions concerning the phosphate bond energy in nucleic acid, since a high energy bond would be required in order to phosphorylate glucose. The structure of nucleic acid proposed many years ago by Levene does not readily lend itself to the suggestion that some of the phosphate bonds may be of the high energy group. Recent studies by Gulland, however, indicate that the structure of nucleic acid is far more complex than that suggested by Levene. It is not necessary to assume, however, that energy required in a com-

plex transphosphorylation process must all be supplied by the phosphate bond initially split. In fresh frozen sections metabolic processes involving endogenous substrates are presumably still active, and may supply the required energy.

Finally it should be pointed out that, if, as we have indicated the dephosphorylation of nucleic acid under the conditions of our experiments involves a complex series of transphosphorylations, then the histochemical localization must refer to the final, not the first link in the chain.

II. EFFECT OF MITOTIC INHIBITORS

In a series of reports from this laboratory, the rat's corneal epithelium has been shown to be an exceedingly favorable test object for the study of mitotic activity (19, 20, 21). A large number of agents have been found which delay or inhibit the onset of mitosis in this tissue. The present investigation was undertaken to explore one possible biochemical mechanism of mitosis inhibition. The studies of Caspersson (22) have shown that the prophase of mitosis is characterized by a very active synthesis of desoxyribose nucleic acid. It is apparent from Caspersson's work that a cell unable to accomplish the normal synthesis and breakdown of this nucleic acid would be unable to undergo a normal mitotic division. It follows that some agents which inhibit mitosis may conceivably produce this effect through inhibiting desoxyribose nucleic acid synthesis.

The suggestion that this is the mechanism of mitosis inhibition by ionizing radiation has in fact been advanced by Hevesy (23). Using radioactive phosphorus as tracer, Hevesy measured the turnover of nucleic acid phosphorus in rats before and after exposure of the animals to X-radiation. He found that following exposure, the phosphate turnover in ribose nucleic acid was normal, but in desoxyribose nucleic acid was markedly reduced. While these results are strongly suggestive of the conclusion which Hevesy sought to draw from them, they cannot be taken as conclusive since, if desoxyribose nucleic acid synthesis should occur normally only during mitosis, then any inhibitor of mitosis should reduce the turnover of desoxyribose nucleic acid phosphate irrespective of the precise point at which the inhibitor interrupts the mitotic cycle, so long as that point anteceded the normal phase

of desoxyribose nucleic acid synthesis. Moreover, Brues (24) has recently reported that in the peculiar "mitotic death" which is seen in some cells after very heavy doses of X-radiation, there is an increase in Foelgen positive material, presumably desoxyribose nucleic acid, in the dying cell. These cells, exposed to ionizing radiation in doses far above those required to delay normal mitoses still are able to synthesize Foelgen positive material.

Bodenstein (25), applying Hevesy's argument to the nitrogen mustards, found that amphibian larvae after exposure to these agents showed much smaller gains in desoxyribose nucleic acid than did normal controls. The interpretation of these data involves the same difficulties as those of Hevesy. Moreover, the nitrogen mustards appear to have an even greater propensity to produce "mitotic death" with increased Foelgen positive intranuclear material than has ionizing radiation.

In view of these contradictory implications, it seemed desirable to attack this problem by a definite assay of some enzyme potentially concerned in nucleic acid synthesis, and to pursue the study, if possible, by a histochemical technique which might reveal differences between cells normally capable of mitotic division and cells no longer capable of this activity. Since there are no doubt many different enzymes involved in nucleic acid synthesis, failure to find a particular one of these enzymes inhibited after exposure to a mitosis inhibiting agent would not disprove Hevesy's hypothesis. A positive experimental result would, however, be conclusive. This argument led us to attempt to develop the histochemical technique for the demonstration of phosphatase activity with ribose and desoxyribose nucleic acids as substrates, and to apply this technique to rat's corneas with and without previous exposure of the tissue to agents capable of inhibiting mitosis in this tissue.

The histological technique has been reported in the previous section of this paper. In a series of experiments the effect of various mitosis inhibitors on the nucleic acid phosphatase reaction was tested. Rat's eyes were exposed in vivo to heavy doses of X-radiation, ultraviolet, and the nitrogen mustard $CH_3N(C_2H_4Cl)_2HCl$. Eyes were removed at various times after exposure and frozen sections prepared from them were compared in respect to nucleic acid phosphatase activity with

sections from the unexposed opposite eye of the same animal. No difference in activity was found between the treated and the control eye for either ribose or (depolymerized) desoxyribose nucleic acid in the presence or absence of glucose and KCl.

Tests were made in which cocaine, adrenalin, or arsenic were added to the substrate solutions. No effect was found on the nucleic acid phosphatases of concentrations of cocaine or adrenalin far in excess of that required to inhibit mitosis. High concentrations of arsenite $\frac{M}{200}$ produced a partial inhibition of activity for both nucleic acids. The concentration of arsenite required for this effect was, however, considerably greater than that required to produce inhibition of mitosis in vivo. It is to be concluded that none of these agents inhibit mitosis through inhibition of the nucleic acid phosphatase.

CONCLUSIONS

Though the nucleic acid phosphatase system appears to be a complex and integrated mechanism, our experiments reveal no evidence of inhibition or disorganization of this system by those agents that are notorious for their capacity to inhibit mitosis. It is concluded that the particular mitosis inhibitors which we have tested do not inhibit mitosis through inhibiting or disorganizing the nucleic acid phosphatase system.

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MEETING OF THE JOHNS HOPKINS MEDICAL SOCIETY

MONDAY, MARCH 14, 1949—8:15 P.M.

HURD MEMORIAL HALL, THE JOHNS HOPKINS HOSPITAL

Dr. Follis: The first paper on this evening's program is by Drs. Walker, Marshall, and Johnson, and the title is

ELECTROCORTICOGRAPHY

BY DRS. A. E. WALKER, H. C. JOHNSON AND C. MARSHALL

Electrocorticography is the process of recording cortical potentials directly from the exposed brain at the time of operation, using cortical electrodes and an electroencephalograph. This technique has a number of applications.

Epileptic foci which can be localized only to a certain region of the brain by the use of scalp electrodes can be sharply localized to a definite area of cortex with cortical electrodes. Epileptic foci which do not show spontaneous spiking may be localized by the intravenous injection, or local application to the brain, of metrazol, together with simultaneous recording of the cortical electrical activity. The effectiveness of cortical resection of a focus is also demonstrated by electrocorticography.

Cerebral neoplasms not infrequently lie subcortically and are not apparent at the time of exposure of the brain. A tracing of the exposed cortex may show slow waves arising from the brain overlying the tumor.

Electrocorticography is a valuable tool in the study of the physiology of the cerebral cortex. An example of this is the outlining of sensory areas of the cortex by the recording of evoked potentials. This technique has been used to date only for the study of the somato-sensory areas, but it should have a much wider range of application in the future.

Dr. Follis: Dr. Walker and his collaborators' paper is open for discussion. If there is no discussion of Dr. Walker's paper the next paper will be by Dr. Elliot Newman and Drs. Kattus, Sinclair-Smith, Genecin, Sisson, and Monge. The title is "Studies on the Function of the Kidney in Cardiac Failure."

STUDIES ON THE FUNCTION OF THE KIDNEY IN CARDIAC FAILURE

BY DRS. ELLIOT NEWMAN, KATTUS, SINCLAIR-SMITH, GENECIN, SISSON AND MONGE

The purpose of this paper is to present and discuss some examples of studies by which we have attempted to describe the functional alterations of the kidney in cardiac failure.

Our studies are made in an attempt to describe the mechanism of the salt retention by the kidney by correlating the changes in renal function with the clinical and metabolic condition of the patient and to attempt to determine some of the factors bearing upon salt retention by a study of the effect of stress and drugs upon the kidney in normal subjects and patients with cardiac failure.

Our first study was of a 37 year old colored woman with marked congestive failure due to rheumatic mitral and aortic valvular deformities. She had the murmurs of mitral stenosis and insufficiency and of aortic insufficiency. There was no evidence of active rheumatism. When admitted to the hospital she was markedly orthopnoeic and dyspnoeic at bed rest with distended neck veins and peripheral edema.

The first 40 days during weight loss, a fall in venous pressure and symptomatic improvement were associated with a rise in renal plasma flow and fall in filtration fraction to normal. There was no significant change in the glomerular filtration rate. Thereafter the patient was at home and deteriorated again. She returned to the dispensary and was observed to have increased weight and venous pressure with markedly reduced renal plasma flow with the same glomerular filtration rate.

The glomerular filtration rate was quite constant and within normal limits for this patient throughout the entire period of observation. Her normal glomerular filtration rate according to the standards of Homer Smith would be 100 cc./min. The range of her determinations of glomerular filtration rate was 85 to 115 cc./min.

During the weight loss, metabolic studies were performed on this patient. No medication except a maintenance dose of digitalis was given.

Loss of sodium and chloride were parallel to the drop in weight and venous pressure. At first the intake of sodium chloride was low (2 gm. of sodium chloride), later the intake was raised by an additional 5 gm. of sodium chloride. The increased intake had very little effect on the general course of sodium chloride balance, the kidney rapidly excreting the extra salt. All these changes in sodium and chloride balance and total output took place without any significant variations in the glomerular filtration rate.

We conclude from the facts so far that the regulation of sodium chloride excretion by the kidney in congestive failure is not correlated with changes in glomerular filtration rate during recovery, and that a normal glomerular filtration rate can be maintained.

Our second case study was a 30 year old man with a history of rheumatic heart disease. He had dyspnoea on slight exertion with minimal cyanosis. He had minimal but definite edema of the feet and an elevated venous pressure to 160 mm. His heart was enlarged to the left and right and the rhythm was auricular fibrillation. Enlargement of the left auricle and calcification of the mitral valves were demonstrated by x-ray. He had been taking digitoxin, 0.1 mgm. a day, for several months, and this was continued with no other medication.

Throughout the period of balance study no significant change in these renal func-

tions occurred. The initial weight loss was accompanied by a fall in venous pressure from 160 mm. to normal, and there was marked negative balance of sodium and chloride. Following this there was retention of sodium and chloride for several days. The total amount retained during this period was nearly equal to that lost previously. However, no change in weight accompanied this retention. During this phase the intake was changed from 3 gm. of sodium chloride to 7 gm. The extra salt was promptly excreted with no alteration in the balance pattern. Later the patient was allowed up and given some exercise during the day, which was associated with some further sodium chloride retention. No significant gain or loss of potassium or nitrogen occurred in this patient.

We conclude from this study that a glomerular filtration rate 50% of normal and a renal plasma flow diminished to ½ normal did not prevent loss of edema and superfluous body sodium nor was there any correlation between either glomerular filtration rate or renal plasma flow and sodium chloride balance. This patient had persistent renal ischemia throughout his observation and was able to excrete stored body sodium as well as extra sodium added to his intake. The renal blood flow did not increase with compensation as in the first patient.

We conclude, then, that the function of the kidney in cardiac failure may be affected in many ways and that the metabolic effects on the tissues other than the kidney of inadequate output of the failing heart, may be complicated and profound. The patient must be studied not only at rest but under the stresses of daily activity. Only then can we obtain an integrated picture of the many inter-related factors involved in the production and retrogression of the congestion and edema. We have seen that ischemia of the kidney occurs which is presumably a reflection of inadequate cardiac output. This may cause some retention of sodium and chloride by diminishing glomerular filtration rate. But the ultimate responsibility rests with the specific selective reabsorptive mechanisms of the renal tubule cells whose activity must be sensitive to other factors beside the reduction in the amount of fluid presented to them by the diminished circulation. The effects of posture, exercise and humoral substances and the renal nerves have as yet to be adequately determined, and the nature of the metabolic injury to other tissues is a relatively unexplored phase of the problem.

Dr. Follis: Dr. Newman and his collaborators' paper is open for discussion.

Dr. Futcher: I think we are all interested to see Dr. Newman throw the spotlight on the function of the renal tubules in this very important problem of cardiac failure. I was particularly interested to hear him mention the possible relationship between tubular function and the renal nerves. Two years ago in St. Louis my colleagues, Dr. Kriss and Dr. Goldman, and myself more or less by mistake became concerned with the influence of the renal nerves upon renal tubular function. We had been interested in the effect of removing the left adrenal upon the function of the left kidney and might have saved outselves some trouble if we

had paid a little more attention to a study of Dr. E. K. Marshall's in 1919, which was mentioned by Dr. Newman. He demonstrated that the effect of removal of the left adrenal upon function of the left kidney could be shown only insofar as one injured the nerves to the left kidney, particularly the splanchnic nerve, inadvertently during the process of adrenalectomy. We can report to Dr. Marshall that the situation hasn't changed in the dog's kidney in the last twenty years, and we found that we could explain our findings after adrenalectomy as chiefly due to the injury we caused to the nerves during our operation. Our experiments consisted in putting cannulas in both ureters of anesthetized dogs and determining the effect of unilateral adrenalectomy upon the excretion of chloride and water. During the course of the observations, we obtained some evidence which was not conclusive but suggested that cutting the left splanchnic nerve increased the excretion of water and chloride by the left kidney, by direct effect on the tubule consequent to cutting the nerve. For although there was increased filtration of water and chloride secondary to the cutting of the nerve, this increased filtration was not enough in certain instances to explain the increased excretion of water and chloride. It is possible that splanchnicectomy interfered with nervous impulses to the renal tubules of these anesthetized dogs and resulted in decreased reabsorption of water and chloride by the tubules and in an increased excretion. There were alternative explanations for these findings and we could not be sure about the mechanism.

Now these experiments were in anesthetized dogs and I think it extremely questionable whether their possible implications can be transferred to the situation in man. I agree with Dr. Newman it will be of interest to find out about the function of these nerves in their relationship to the renal tubule of man.

Dr. Andrus: As if cardiac failure weren't complicated enough from the circulatory standpoint, it has now become a metabolic abnormality. If I recall the circulation of the kidney correctly, the efferent flow from the glomerulus represents a large part of the flow to the tubule. How far does the reduction of the circulation to the tubules, or does it in any part, account for the abnormalities which might be observed?

Dr. Follis: Is there any other discussion of Dr. Newman's paper. Dr. Newman.

Dr. Newman: We tried to ascribe the control of sodium and chloride excretion upon one circulatory factor after another in the kidney and every time we have been upset by the next case we studied. In the first case studied, there seemed to be a correlation between the metabolic balance of sodium and the renal plasma flow. In the second case, there was none, and since that time we have seen other cases with no correlation between renal ischemia and the metabolic balance of sodium and chloride. For a time we thought that the high filtration fraction had something to do with salt retention because fluid filtered through the glomerulus

at a high pressure means that the plasma reaching the tubules would be more concentrated and might increase the degree of reabsorption of salt and water by the tubules. But, we have seen in the second case the filtration fraction did not change although major changes in the sodium chloride balance occurred.

Dr. Follis: Is there any other discussion? If not, the meeting is adjourned.

BOOK REVIEWS

These reviews represent the individual opinions of the reviewers and not necessairly those of the members of the Editorial Board of this Bulletin

Acclimatization in the Andes. By CARLOS MONGE. *Baltimore, The Johns Hopkins Press*, 1948, 130 + XIX pp.

The author, who is Director of the Institute of Andean Biology and quondam Dean of the Medical Faculty of the University of San Marcos, Lima, Peru, is known internationally for his broad studies in the biology of man and animals living on the high plateaus of the Andes. This little book, crowded with generous quotations from the records of early Spanish explorers, conquistadores, priests and rules, is an engrossing description of the "bio-climatic determinism" which shaped the course of Andean native societies. Compelling evidence is furnished to demonstrate that much social and labor legislation of both the Incan empire and the Spanish viceroys was directed toward preserving the racial acclimatization of Andean natives, won over many generations. Dr. Monge regrets the incongruity of the fact that, "The Republic is still unaware of the problem." This book is recommended highly as an example of the fruitful results and promising future of studies which combine biological, geographical and sociological technics.

J. L. L., Jr.

Aviation Medicine in Its Preventive Aspects. By JOHN F. FULTON. *Oxford University Press*, 1948, VIII, 174 pp.

These essays comprise the Heath Clark Lectures delivered by the author in 1947 at the London School of Hygiene and Tropical Medicine. The five lectures are devoted to the important problems of altitude, decompression sickness, explosive decompression, acceleration and crash. In his familiar enthusiastic and felicitous style, Dr. Fulton presents phases of the historical background, the classical experimentation and the recent investigations in these fields. It must be emphasised that these attractive essays are by no means complete reviews of the topics listed. The reader who has had experience in this special field may note and perhaps regret that the form of these lectures has prevented inclusion of a number of important and fundamental studies executed during World War II. These omissions, necessitated perhaps by the ambitious scope of these lectures, do not detract from the clear and exciting story which Dr. Fulton tells so well.

J. L. L., Jr.

Cancer of the Esophagus and Gastric Cardia. Edited by GEORGE T. PACK, B.S., M.D. *St. Louis, C. V. Mosby Co.*, 1949.

This monograph is a symposium on the diagnosis and treatment of esophageal and gastric cancer originally published in the June, 1948 issue of *Surgery*. Arti-

cles are included by such outstanding authorities, among others, as Garlock, Ochsner, and Sweet. As Pack points out, the progress made in the treatment of cancer of the esophagus is one of the notable achievements in surgery of the past decade. This book should be of considerable interest to students of surgery and surgeons who are working in this field.

E. S. S.

Doctors of Infamy. By ALEXANDER MITSCHERLICH. 172 pp. $3.00. *Published by Henry Schuman, Publisher, 20 East 70th St., New York 21, N. Y.*

This does not make for nice, pleasant reading. The subject matter does not allow it. Yet, it should be read by all who are interested in experimental medicine and by all who hope some time in some small way to add to the knowledge of diseases of man.

This is a factual, documented account of the cruel and useless experiments performed by some German "physicians" under the Nazi regime. Some of them would have been classified as "reputable scientists."

A number of us heard of the indirect evidence during the war that various antirickettsial drugs and vaccines were being tested on cases of epidemic typhus caused by inovulating typhus rickettsia into man. Here, however, is the entry (p. 44) from a Dr. Ding's journal dated April 24, 1943:

"Therapy tests, acridine granulate (A-Gr-2) and rutenol (R-2). To conduct these therapy tests with acridine granulate and rutenol, thirty persons (fifteen each) and nine controls were intravenously injected, each with 2 cc. of fresh blood from typhus patients. All the test persons contracted very virulent cases of typhus."

The rickettsia were maintained at full virulence in man by continued passage in man. Some three to five "carriers" were inoculated per month. Their mortality was probably about 95%.

The comparative efficacy of different vaccines (unfortunately these are called "serums" in the text) was tested. Louse vaccine Weigl yolk sac (Cox-Gildemeister-Haagen method) and several others were used with ten persons as controls. The whole group was then infected in the presence of Prof. Gildemeister. The overall test of the efficacy of the vaccines was as follows: Of some 90 controls, 40 died, whereas of 383 protected by vaccine, 66 died. This is a significant reduction of mortality and might be quoted as useful information. However, such information was obtained by Allied forces without recourse to such direct, crude experimentation. Furthermore the relative value of the different vaccines is not established.

A series of experiments on sulfonamide, bone grafting, cellulitis, and mustard gas were carried out under the direction of the Professor of Orthopedic Surgery at the University of Berlin. These involved the direct inoculation of bacteria, tiny fragments of wood and glass into wounds. These experiments were not considered adequate by the S.S. because they did not sufficiently approximate the conditions at the front, and the next group of experiments were ordered with the

stipulation that "it would be necessary to inflict actual gunshot wounds on the patients."

On page 71, a case history begins,"November 11, 1942: At 1800 hours, under the designation 'purolin', the patient received an injection of 1 cc. of pus in which numerous streptococci chains had been observed under the microscope. The injection was administered on the inside of the left thigh close to the abductor canal. Late at night the patient complained of severe headache and drawing pains in the left thigh.

Many other types of "experiments" are described and documented: Exposure to cold, drinking of sea water, low pressure.

This book is a valuable record, which cannot be read without its making the reader think a great deal about the purposes of experimental medicine.

F. B. B.

Experimental Immunochemistry. By ELVIN A. KABAT and MANFRED M. MAYER. *Charles C. Thomas, Springfield, Ill.*, 1948, $8.75. XV + 567 pp.; 88 figures.

The authors have undertaken the important task of describing the experimental methods employed in immunochemistry, with emphasis upon quantitative chemical methods. Part I deals in detail with methodology, and Part II with the applications and uses of quantitative immunochemical procedures. Subsidiary to these sections is the material occupying the second half of the book. This contains more or less detailed descriptions of certain chemical and physical methods and special procedures often used by the immunochemist (Part III), and directives for the preparation of selected variety of substances (Part IV).

Although *Experimental Immunochemistry* is primarily for the laboratory, it is more than a manual of procedures. The discussions and comments, and the selcted references, provide some of the common ground for the overlapping interests of the immunologist and the chemist.

As Dr. Michael Heidelberger states in his introductory comment, a complete treatise on immunochemistry is yet to be written. In the meantime, Kabat and Mayer's book may earn for itself a useful position alongside the works of Landsteiner, Marrack, and Boyd which have become established as indispensable aids to the student in this important field of knowledge.

B. C.

Nursing Care of Neurosurgical Patients. By ROLAND M. KLEMM. *Springfield, Illinois, Charles C. Thomas*, 1949, xii + 117 pp., 62 figures.

This book presents a concise account of a neurosurgeon's view of nursing care for his patients. The text covers the clinical phenomena of neurological disease with which the nurse should be familiar. The various conditions amenable to neurosurgical operative treatment are briefly discussed. A section on operative technique, illustrating the instruments and operating room arrangements for various surgical procedures, is particularly valuable. An appendix describing

special nursing techniques such as enemas, packs, etc., will be of interest to many student nurses.

The technical information of the book, aided by clear illustrations, makes a valuable background for a better understanding of the problems involved in the care of the neurosurgical patient. But the student nurse or recent graduate unacquainted with neurosurgical terminology and practice may be confused by the text. For example, on the opening page the author presents an illustration of the layers of the scalp showing the "skin, superficial fascia, aponeurosis, subaponeurotic connective tissue, pericranium, subpericranium, skull and dura mater." But in the text he refers to "the galea" without indicating that the galea aponeurotica is synonymous with "aponeurosis."

In a textbook, absolute accuracy on well established points, is a necessity for otherwise the student integrates in his thinking misinformation which may never be eradicated. Yet on page 5 "Anterior to the cerebellum is the medulla and in direct continuity and below is the pons. . . . Immediately below the pons is the spinal cord." Loose thinking probably accounts for a number of colloquialisms used in the book. "The aphasic structures lie in an indefinite delimited area. . . ." (p. 4). "The glossopharyngeal and vagus are functionally closely related. The former influences taste on the posterior third of the tongue, acting soft palate noted as well as gag reflex. Aphasia disappears and hoarseness may be vagus involvement. Special examination of vocal cords may be indicated. Vagus: pulse rate, respiration" (p. 26). Misspellings or typographical errors are rather common.

Written from a surgeon's viewpoint it is perhaps understandable that details of nursing care are underemphasized, but in a book having a title such as this one, it might be expected that some discussion of nursing principles be given rather than the subject dismissed as it is on p. 60 "In thoracic sympathectomies, the same as any chest case." As a further elaboration of this theme, the book does not indicate that a patient is more than a neurosurgical case. Certainly today, the individualization of nursing care to meet the demands of the patient's personality is an important factor in both pre- and post-operative surgical therapy.

The book is well illustrated with both black and white and colored drawings, in this regard, being superior to most nursing textbooks. The general appearance of the book is pleasing and a tribute to the publisher.

The book should be a useful reference for the nurse working with neurosurgical patients either on the ward or in the operating room, but it cannot be recommended as a textbook for the student nurse.

A. E. W.

Obstetrical Analgesia and Anesthesia. By FRANK F. SNYDER, M.D., Associate Professor of Obstetrics, Associate Professor of Anatomy, Harvard School of Medicine. *W. B. Saunders Company*, 1949.

For the reader who wishes documented evidence of the hazards of parturition, "Obstetrical Analgesia and Anesthesia" by Frank Snyder provides far more;

for the physician who wishes to apply this information into practical technics of safe control of obstetric pain, this book provides far less. The author, from a wide experience in three medical schools over a period of many years, presents the summations of his experiences and experiments upon the animal fetus in a well written compendium on the physiology and pathology of the respiratory system during the transition from the intrauterine to the extrauterine existence. This section comprises more than half of the book. It records the stillbirth and neonatal death rates in rats, rabbits, guinea pigs, and man, as related to the incidence of intrauterine pneumonia, atelectasis, asphyxia, and pharmacologic intoxication.

Doctors Snyder and Rosenfeld of the Department of Pharmacology of the Johns Hopkins University performed an ingenuous experiment for prolonging the intrauterine gestation period of the rabbit. This was accomplished by the injection into the pregnant rabbit near term with urine from a pregnant human. Such injection initiated ovulation and formation of secondary corpora lutea. Such hormonal influence delayed labor by a week or more. These maternal animals were then prepared by transections of their spinal cords. Abdominal laparotomy permitted the pregnant uterus with intact blood supply in tact to come into view in a warm saline bath. Fetal rabbit movement, respiratory patterns, and nerve stimulus reactions could for the first time be accurately recorded. Incision of the uterus permitted the baby rabbits to come out into the saline bath while still attached to their placental circulation. Under such conditions it was possible to determine the effects of all sedatives, narcotics, and general anesthetic drugs on fetal physiology. It was found that the fetal respiratory system was most delicately sensitive to all of these agents. Morphine, nitrous oxide, barbiturates, paraldehyde, chloroform, and ether, were most depressing.

While such experiments presented interesting information for the fetal rabbit, it could definitely be considered inconclusive as a theoretical pattern of human reaction, since the fetal rabbit at birth is very much less developed to correspond to approximately the sixth intrauterine month of the human fetus. It is well known that fetuses in early months of gestation are much more resistant to anoxia than are those nearer term.

The chapter on respiration before birth embodying Doctors Snyder's and Rosenfeld's original work is the classic chapter of the text. The chapter on intrauterine pneumonia, while interesting, is in no way related to the subject of obstetrical analgesia and anesthesia. The chapters on atelectasis and asphyxia are of greater importance.

The second half of the book on the treatment of pain during labor is a 170-page inaccurate compilation of the literature, which presents good evidence that the author is neither anesthesiologist nor an experienced obstetrician with adequate experience with the drugs he describes. No physician who reads these chapters could treat the pain of labor and delivery with security of accurate information. He would have been so disarmed with the dangers of obstetric amnesia and analgesia presented in the first half that he would be confused and alarmed at the admonitions of the second half. Entirely too much space was devoted to mor-

phine, with and without scopolamine combinations, and entirely too little space was devoted to the barbiturates and the local anesthetics. An astounding omission was no mention of the anatomical pathways carrying the pains of parturition. No attention was paid to the control of pain and fear mechanisms as postulated by Grantley Dick Read. No illustrations were used to simplify understanding of complicated techniques. Only three lines were devoted to saddle block spinal anesthesia—a technique that is certainly being used to deliver 8 to 10 per cent of hospital babies. The evidence presented on the use of demerol is inconclusive. It is regrettable that such splendid fundamental work on the physiology of the newborn and the intrauterine fetus should be camouflaged under the nondescriptive title, and further deprecated by an outdated second half of the book.

Nevertheless, this text should be read by every obstetrician and pediatrician. Information recorded within it is of great importance in helping to determine some of the analgesia-anesthesia factors in hospital infant mortality conferences. It is hoped that the publishers of this text will rename it, and that the author will remove from it the insubstantial portions which should not interfere with the wide acceptance of the contributions made herein.

R. A. H.

Preoperative and Postoperative Care of Surgical Patients. By HUGH C. ILGENFRITZ, with foreword by URBAN MAES. *St. Louis, C. V. Mosby Co.*, 1948.

The reviewer can appreciate what an immense amount of study and hard work the author put into the production of this almost encyclopedic book. Surgeons, surgical interns, and those in residency training will find much useful information in the book, and it would be good practice to have a copy on the shelf in each ward office. The author presents excellent discussions of the physiologic bases for the usual therapeutic measures, and adequately presents the details of the techniques of these measures. The pathogenesis of surgical complications is outlined, and means of prevention as well as for cure are given. A most helpful feature is the inclusion in each discussion of reference to recent original articles in the literature pertinent to the subject. When controversial matters are discussed, such as the therapy of postoperative thrombosis of veins, or early ambulation, justice has been done to the views of both sides, although the reader may sense that the author's sympathy is on the side of conservatism.

The volume is well printed and contains numerous clear illustrations. The author considers in detail the postoperative care of patients who have had thoracic, abdominal, gynecologic, and vascular surgery, but does not discuss the postoperative care of neurosurgical, otolaryngological, urological, or orthopedic patients; the treatment of burns, however, is included. Perhaps the book would be of more value to interns if all the surgical specialties were included. All in all, the reviewer believes this compendium of modern knowledge to be a useful and worthwhile contribution.

E. S. S.

Psychiatry in General Practice. By MELVIN W. THORNER, M.D., D.Sc. *W. B. Saunders Co., Philadelphia and London,* 1948.

In discussing the purpose of the book, the author stresses the need for psychiatric publications which are relatively free from highly technical psychiatric "lingo" but sufficiently informative to be of practical use to the physician in the care of patients with psychiatric disorders. In organizing the book, he has all but abandoned standard classification by the use of such adjectives as "confused," "suspicious," "anxious," "queer," etc. attached to the noun "people." This procedure is designed to lead the reader to look upon mental illness as a quantitative rather than a qualitative variable. Yet, it is also likely to be misleading. For example, neurotic reactions are discussed under "anxious people" even though it is a well-known fact that many of these people are not at all anxious. To call psychopathic personalities "queer and twisted people" adds little to the understanding of their personality disorders while it might confuse those readers who know that "twist-reactions" are frequently referred to as "para"-disorders. The greatest asset of the book is the reporting of the case abstracts. Rarely is there to be found in psychiatric literature a more colorful description of the lives of people which, at times, becomes so strong as to appear almost unbelievable. The author's literary skill is undeniable and makes up for the excessive length and occasional irrelevance of the details reported on. Unfortunately, the book's weakness is to be found in the chapter on therapy. Even though examples of brief psychotherapeutic methods and their successes are offered, the reader learns little about when to apply them. More specialized techniques are mentioned as they concerned physical methods, but one is left with the impression that the author considers intensive psychotherapeutic measures too theoretical to be of interest for the general practitioner.

The author's effort in writing the book is unquestionably a step in the right direction. It could have been more successful had more space been devoted to the illustration of treatment of patients suffering from psychiatric disorders which a competent general practitioner may be called upon to carry out. Such a text will probably have to be written by the psychiatrically trained practitioner himself rather than the psychiatrist whose experience is necessarily limited by his specialization and restricted to the more complex problems of the field.

E. A.

The Digestive Tract in Roentgenology. By JACOB BUCKSTEIN, M.D. *J. B. Lippincott Co., Philadelphia,* 1948. Pp. 889.

As a reference text for students and those practitioners not personally engaged in the roentgen diagnosis of gastrointestinal disease, this volume is a worthy successor to its eminent predecessor of several decades ago. Each of the anatomical divisions of the alimentary tract is subdivided into chapters dealing with the more significant lesions of that structure. Discussion of these topics is largely from the viewpoint of roentgenographic diagnosis. Those techniques deemed most valuable by the author are thoroughly described.

Throughout the work, detailed case reports with accompanying illustrations of excellent quality are employed liberally. Frequently however, the inclusion of such profuse clinical data is at the expense of vital considerations; etiology, pathology, and particularly differential diagnosis. A notable example in this regard is the failure to emphasize the frequent association of pernicious anemia and gastric carcinoma.

Of major importance to the gastroenterologist are the enigmas posed by antral spasm simulating carcinoma and colonic diverticulitis suggesting neoplasm. To the reader who utilizes roentgen methods, omission of such problems by the author is disappointing, in view of his rich experience. Those unfamiliar with these problems may quite erroneously assume that the radiological features of these disease processes are characteristic. Nevertheless, Dr. Buckstein's materials are a meritorious addition to the medical literature and deserve careful perusal.

M. W. D.

The Mechanism of Abdominal Pain. By V. J. KINSELLA. *Sydney Australasian Medical Publishing Company Limited*, 1948.

In this monograph the author, a well-known surgeon of Sydney, Australia, attempts to establish the neuro-anatomical basis for the interpretation of pain arising from physiologic or pathologic disturban es of the intra-abdominal viscera. He believes "that the mechanism for splanchnic pain does not differ essentially from that for somatic pain." His theories and conclusions are based, in part, on clinical studies of patients upon whom he has carried out surgical procedures under local anesthesia. In general, however, the author's approach is largely historical and philosophical.

The reviewer sympathizes with those who would render more accurate the diagnosis of intra-abdominal pathology by the interpretation of the clinical complaints and signs. To this difficult field contributions are most welcome.

E.S.S.

The Renal Origin of Hypertension. By HARRY GOLDBLATT, M.D., C.M. *Published by Charles C. Thomas*, 1948, 126 pages.

As the investigator who devised the method and performed the experiments forming the basis for the modern concept of the kidney's rôle in hypertension, Dr. Goldblatt writes with preeminent authority. In this monograph he summarizes his own work and that of others, integrating the various pieces of evidence into a carefully drawn picture of experimental renal hypertension. Figures illustrating a number of the fundamental experiments first published in various journals between 1934 and 1944, are reproduced here. Several chapters are devoted to the constituents of the humoral mechanism, and in the final chapter a number of similarities and certain differences between human essential and experimental renal hypertension are pointed out. This small volume is a clear exposition of the essential theory of the renal origin of hypertension and serves as an excellent point for orientation toward the problem. It is not a detailed analysis of all the work

related to that subject, nor is an extensive bibliography included, but the sources from which more complete information may be obtained are indicated.

C. B. T.

The Rh Blood Groups and Their Clinical Effects. By P. L. MOLLISON, A. E. MOURANT and R. R. RACE.

This small volume provides an excellent summary of present knowledge of the Rh factors and their clinical significance. The authors, who have made important contributions in this field, are well qualified to discuss the subject. Detailed information concerning the classification of the Rh groups, their clinical significance and method of Rh testing is given in concise form. The material is presented with remarkable clarity and brevity, considering the complexity of the subject.

C. L. C.

Your Child or Mine. By MARY LOUISE HART BURTON. In collaboration with SAGE HOLTER JENNINGS. *Published by Coward-McCann, Inc.*

This book describes the history of five children representing the five types of cerebral palsy: namely, the spastic, the athetoid, the ataxic, and the rigidity and tremor. It illustrates the problems both physical and psychological which occur in these types and does differentiate the problems as they show up in these different types. This book is valueable as an informational source to parents and others who are working with these children.

It is non-technical and accurately written.

W. M. P.

BOOKS RECEIVED FOR REVIEW

Atlas of Peripheral Nerve Injuries. By WILLIAM R. LYONS, Ph.D., and BARNES WOODHALL, M.D. *Published by W. B. Saunders, Phila.* 339 pp. $16.00.

Aviation Medicine. By JOHN F. FULTON. 174 pp. $3.50. *Published by Oxford University Press, 114 Fifth Ave., New York 11, N. Y.*

Cardiac Catheterization in Congenital Heart Disease. By ANDRE COURNAND, JANET S. BALDWIN and AARON HIMMELSTEIN. *Published by the Commonwealth Fund, 41 East 57th Street, New York 22, N. Y.* 108 pp. $4.00.

Child Psychiatry. Enlarged Second Edition. By LEO KANNER. 752 pp. $8.50. *Published by Charles C. Thomas, Springfield, Illinois.*

Clinical Case-Taking. By GEORGE R. HERRMANN, 4th edition. 240 pp. $3.50. *Published by C. V. Mosby Co., St. Louis, Missouri.*

Current Therapy 1949. By HOWARD F. CONN and a large list of Consulting Editors. *Published by W. B. Saunders, Phila.* 672 pp. $10.00.

Doctors of Infamy. By ALEXANDER MITSCHERLICH. 172 pp. $3.00. *Published by Henry Schuman, Publisher, 20 East 70th Street, New York 21, N. Y.*

Handbook of Diseases of the Skin. By RICHARD L. SUTTON and RICHARD L. SUTTON, JR. 749 pp. 1057 illus. $12.50. *Published by C. V. Mosby Co., St. Louis, Missouri.*

Hindu Medicine. By HENRY R. ZIMMER. 201 pp. $4.00 *Published by The Johns Hopkins Press, Homewood, Baltimore 18, Md.*

Introduction to Human Anatomy. By CARL C. FRANCIS. *Published by C. V. Mosby Co., St. Louis 3, Missouri.* 470 pp. $5.50.

Neurological and Neurosurgical Nursing. By GUTIERREZ-MAHONEY and CARINI. *Published by C. V. Mosby, St. Louis, Missouri.* 516 pp. $5.75.

Nursing Care of Neurosurgical Patients. By ROLAND M. KLEMME. *Published by Charles C. Thomas, Publisher, 301 E. Lawrence Ave., Springfield, Illinois.* 142 pp. 62 figures. $3.00.

Operating Room Technique, 2nd edition. By EDYTHE LOUISE ALEXANDER, R.N. 765 pp. $10.00. *Published by C. V. Mosby Co., St. Louis, Missouri.*

Pain Syndromes. By BERNARD JODOVICH and WILLIAM BATES. pp. 357. $6.00. *Published by F. A. Davis Co., Phila. 3, Pa.*

Pharmacologic Principles of Medical Practice. By JOHN C. KRANTZ, JR. and C. JELLEFF CARR. pp. 980. $10.00. *Published by The Williams & Wilkins Co., Baltimore, Md.*

Posttraumatic Epilepsy. By A. EARL WALKER. 86 pp. $2.75. *Published by Charles C. Thomas, Publisher, 301 East Lawrence Ave., Springfield, Illinois.*

Practical Aspects of Thyroid Disease. By GEORGE CRILE, JR. *Published by W. B. Saunders Co., Phila.* 355 pp. 101 figures, $6.00.

Safeguarding Motherhood. By SOL T. DE LEE. 135 pp. $2.00. *Published by J. B. Lippincott Co., Phila., Pa.*

Textbook for Almoners. By DOROTHY MANCHEE. *Published by The Williams & Wilkins Co., Baltimore, Md.* 466 pp. $7.50.

The Rh Blood Groups and their Clinical Effects. By P. L. MOLLISON, A. E. MOURANT and R. R. RACE. *Published by His Majesty's Stationery Office, London, England.* pp. 74, 1s. 6d. net.

The Story of Scabies. By DR. REUBEN FRIEDMAN. pp. 468. $7.50. *Published by Froben Press, Inc. 1776 Broadway, New York 19, N. Y.*

Veterans Administration Technical Bulletins, Series 10. Published by the Veterans Administration, Washington 25, D. C.

Campbell's Operative Orthopedics. By J. S. SPEED and HUGH SMITH, vols. I & II. 1643 pp. $30.00. *Published by C. V. Mosby Co., St. Louis 3, Missouri.*

Rheumtic Fever. By SABRA S. SADLER, R.N., B.S. 151 pp. 204 illus. $3.50. *Published by J. B. Lippincott Co., Phila, Pa.*

Vitamin A Requirement of Human Adults: An Experimental Study of Vitamin A Deprivation in Man. Compiled by E. M. HUME and H. A. KREBS. *Published by His Majesty's Stationery Office* 1949, 145 pp. 3s.

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